National Public Health Institute, Department of Microbiology,
Helsinki, Finland

University of Helsinki, Faculty of Medicine, Helsinki, Finland

MOLECULAR EPIDEMIOLOGY OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* IN FINLAND

Saara Salmenlinna

Academic dissertation

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Supervisor
Docent Jaana Vuopio-Varkila, M.D, PhD.
Department of Microbiology,
National Public Health Institute, Helsinki, Finland

Reviewers
Docent Pentti Kuusela, M.D, PhD.
Department of Bacteriology and Immunology,
The Haartman Institute, University of Helsinki, Finland

Docent Mikael Skurnik, PhD.
Department of Medical Microbiology and Molecular Biology,
University of Turku, Finland

Opponent
Alex van Belkum, PhD.
Department of Medical Microbiology and Infectious Diseases,
Erasmus Medical Center Rotterdam, Erasmus University,
Rotterdam, The Netherlands

JULKAISIJA-UTGIVARE-PUBLISHER

Kansanterveyslaitos
Mannerheimintie 166
00300 Helsinki
puh. (09) 47441
fax. (09) 47448238

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsinki
tel. (09) 47441
fax. (09) 47448238

National Public Health Institute
Mannerheimintie 166
00300 Helsinki
phone. +358-0-47441
fax. +358-0-47448238

To my family

Contents

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals.

I. Salmenlinna S, Lyytikäinen O, Kotilainen P, Scotford R, Siren E, Vuopio-Varkila J. Molecular Epidemiology of methicillin-resistant *Staphylococcus aureus* in Finland. Eur J Clin Microbiol Infect Dis 2000;19:101-7.

II. Salmenlinna S and Vuopio-Varkila J. Recognition of two groups of methicillin-resistant *Staphylococcus aureus* strains based on epidemiology, antimicrobial susceptibility, hypervariable-region type, and ribotype in Finland. J Clin Microbiol 2001;39:2243-7.

III. Salmenlinna S, Lyytikäinen O, Vuopio-Varkila J. Community-acquired methicillin-resistant *Staphylococcus aureus*, Finland. Emerg Inf Dis 2002;8:602-7.

IV. Salmenlinna S, Vehkaoja L, Vuopio-Varkila J. Analysis of genetic background of predominant methicillin-resistant *Staphylococcus aureus* in Finland. Submitted for publication.

In addition, some unpublished results are included.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
agr	accessory gene regulator
aux	auxilliary factors for methicillin resistance
AP-PCR	arbitrarily primed PCR
BORSA	borderline resistant *Staphylococcus aureus*
EARSS	European Antibiotic Resistance Surveillance System
FAME	fatty acid modifying enzyme
fem	factors essential for methicillin resistance
HELICS	Hospitals in Europe Link for Infection Control through Surveillance
ICU	intensive care unit
Ig	immunoglobulin
IL	interleukin
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
GlcNAc	N-acetylglucosamine
MurNAc	N-acetylmuramic acid
NCCLS	National Committee for Clinical Laboratory Standards
NINSS	Nosocomial Infection National Surveillance Scheme (UK)
NNIS	National Nosocomial Infections Surveillance System(USA)
MHC	major histocompatibility complex
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin binding protein
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PVL	Penton-Valentine leucosidin
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
sar	staphylococcal accessory gene regulator
SCC*mec*	staphylococcal casette chromosome *mec*
slv	single locus variant
ST	sequence type
TSST 1	toxic shock syndrome toxin 1

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections worldwide, and hospital outbreaks caused by MRSA are common. Unless outbreaks are controlled, MRSA may reside permanently in a hospital environment. Such endemic MRSA are difficult to eradicate, and they most likely further increase the number of infections, the costs, and the length of hospital stays. MRSA strains are commmonly resistant to multiple antimicrobials, which narrows treatment possibilities and changes the spectrum of antibiotics prescribed in hospitals.

This study was performed to analyze comprehensively the Finnish MRSA isolates collected through a nationwide surveillance program since 1992 by various molecular methods. We describe the elaboration of a typing scheme suitable for continuous national surveillance in a country with a low prevalence of MRSA, and analyze the overall trends of MRSA, as well as the proportion of community-acquired MRSA. We especially focus on the characterization of predominant MRSA strains linked to interhospital epidemics and local intrahospital outbreaks. We assess clonality, evolution, association with community acquisition, and molecular traits linked to increased transmissibility.

The baseline number of annual MRSA remained stable in the 1980s and early 1990s. After a peak caused by extensive hospital epidemics in 1994, an increasing trend has been observed. The incidence of MRSA increased from 1.7 in 1995 to 5.0 in 2000 per population of 100,000. Between 1997 and 1999, one fifth of all MRSA isolates were from persons who had not been hospitalized within two years before the date of the MRSA isolation. It was suspected that these isolates had been acquired in a community setting.

A total of 38 epidemic or local outbreak strains were identified between 1992 and 2001. By phage typing, ribotyping, pulsed field gel electophoresis, and multilocus sequence typing, 31 of these strains clustered into eight different clones. Representatives of six clones had multilocus sequence types identical to international MRSA clones, the Brazilian, Iberian, UK EMRSA-16, UK EMRSA-15, New York, and Berlin clones. Together, these clones accounted for 35% of all MRSA isolates in 1997-1999. The remaining two clones, named Joensuu and Mikkeli, showed a molecular epidemiology different from that of the pandemic MRSA clones. The multilocus sequence type of Joensuu is commonly found among international methicillin sensitive *S. aureus* strains, and the Mikkeli sequence type is a single locus variant of another, apparently rare, MRSA type. The Mikkeli clone was the most prevalent MRSA clone in Finland between 1997 and 2001.

Phage typing, ribotyping, and pulsed field gel electrophoresis revealed that three strains were associated with community acquisition.

These strains belonged to the Mikkeli and Joensuu clones, and the third was a triple allele multilocus sequence variant of the Joensuu clone.

Search for epidemicity markers revealed two different populations of MRSA strains. One group contained strains showing *mec* hypervariable region hybridization pattern A combined with a variety of ribotypes and resistance to beta lactam antibiotics only. The majority of these strains were sporadic by nature. The other group contained strains with *mec* hypervariable region hybridization pattern B or C in association with two ribotypes, and resistance to other antibiotic groups in addition to beta lactams. This group contained both epidemic and sporadic strains.

Taken together, these results suggest that two epidemiologically and evolutionarily distinct MRSA populations exist in Finland: 1) global clonally disseminated, often multiresistant strains, and 2) strains sensitive to multiple antibiotics with genetic backgrounds related to methicillin-sensitive *S. aureus* strains. Some strains of the latter population may have recently acquired the *mec* DNA through horizontal transfer, and this may have occured in a community setting as well.

1. INTRODUCTION

Nosocomial infections cause a substantial burden for health and economics worldwide. A nosocomial infection is defined as an infection acquired in hospital, and which is not in the incubation phase on the patient's admission to hospital. However, a nosocomial infection may be caused by a colonizing organism which the patient carried before hospital admission (84). Such endogenous, sporadic infections account for the majority of all nosocomial infections (136, 290, 302). Cross-transmission may lead to epidemics, i.e. an increased number of infections and/or colonizations.

Any microbial group, bacteria, viruses, fungi, or parasites can cause a nosocomial infection, but bacteria are the most prevalent organisms. Among gram positive bacteria, common nosocomial agents include staphylococci, enterococci, streptococci, and *Clostridium difficile*, and among gram negatives, *Escherichia coli*, *Klebsiella sp*, *Enterobacter*, *Proteus*, *Serratia*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp, and *Haemophilus* spp. An additional concern is the emergence and dissemination of nosocomial organisms with increased resistance to antimicrobial agents. Such microbes include methicillin-resistant *S. epidermidis* and *S. aureus*, vancomycin-resistant enterococci, multiresistant and extended-spectrum beta-lactamase-producing gram negative bacteria.

By extrapolating studies performed in Sweden, Norway, and the USA, it has been estimated that approximately 50,000 nosocomial infections occur annually in Finland, and about 1000 persons without a serious underlying disease die of nosocomial infections (2, 17, 99, 155). A recent study showed that the overall rate of nosocomial bloodstream infections in Finland is similar to the rates in England and in the USA, but *S. aureus*, enterococci and fungi are less common as causative agents in Finland (69, 157). The most common bloodstream pathogens in Finland are coagulase negative staphylococci (31%), *Escherichia coli* (11%), and *S. aureus* (11%). Comparison of the number of nosocomial infections between hospitals and countries is difficult for several reasons. First, although definitions of different types of nosocomial infections exist, they may be interpreted in different ways by individual reseachers. Second, comprehensive reporting of nosocomial infections may be difficult to achieve without a substantial resource input. Third, denominator data should be suitable, and gathered in a uniform way (30, 155)

Because of inevitable risk factors related to treatment or to patients, nosocomial infections cannot be totally eradicated. Invasive operations and devices create opportunities for microbes to invade the host tissue, and ingreasingly ill and compromised patients can be treated by evolving techniques and equipment. Risk factors most likely to result in colonization or infection with multiresistant species include advanced age, severity of ill-

ness, inter-institutional transfer, prolonged hospital stay, gastrointestinal surgery, transplantation, exposure to medical devices, and exposure to broad-spectrum antibiotics (236). However, some of nosocomial infections are avoidable, namely those arisen through cross-transmission.

One of the most important pathogens causing considerable morbidity and mortality in hospitals is methicillin-resistant *S. aureus* (MRSA). Owing to expression of an additional penicillin binding protein, PBP2a, with decreased affinity to beta-lactam agents, MRSA is resistant to all beta-lactam antibiotics, including cephalosporins and staphylococcal penicillins (15). Many isolates of MRSA are also resistant to several other antimicrobial groups. Between 1997 and 1998, the SENTRY antimicrobial surveillance program gathered a total of 3981 monthly consequtive bacterial specimens from several types of infections: bloodstream, pneumonia, respiratory tract, skin and soft tissue, and urinary tract infections. Twenty-five European intensive care units were involved. The most prominent organism was *S. aureus*, and 39% of the isolates were resistant to methicillin (76).

The epidemiology of MRSA is changing constantly. Two new stages of MRSA evolution have occurred during recent years: emergence of MRSA strains with reduced susceptibility or with resistance to glycopeptide antibiotics (GISA or GRSA strains, respectively) (31), and community-acquisition of MRSA by persons without known risk factors (29).

The first GISA strain was reported in 1997 in Japan, and thus far about two dozen strains of this type have been reported in different parts of world. The resistance mechanism involves thickening of cell wall peptidoglycan, which walls off vancomycin from the target (110). In July 2002, the first GRSA was reported in the USA in a catheter exit site in a patient with several underlying diseases and multiple courses of antimicrobial therapy. This strain showed minimal inhibitory concentration (MIC) of vancomycin and teicoplanin of >128 μg/ml and 32 μg/ml, respectively, and contained the enterococcal *vanA* gene (31).

Community-acquired MRSA have been isolated from persons with risk factors for MRSA, such as intravenous drug use or previous hospital stays. However, between 1997 and 1999, community-acquired MRSA caused fatal infections in four children without any known risk factors for MRSA (29). Although it is not known whether the community-acquired isolates were originally hospital born, it seems that transmission of MRSA may occasionally occur in the community, and serious infections may develop in previously healthy persons.

This study focuses on molecular epidemiology of MRSA in Finland by analyzing, by various typing methods, the MRSA strain collection gathered by nationwide MRSA surveillance between 1992 and 2001.

The resulting molecular information allows the development of a hypothesis on the evolution of different populations of MRSA, and examined together with epidemiological background information, the characterization of strains associated with community acquisition.

2. REVIEW OF THE LITERATURE

2.1 STAPHYLOCOCCUS AUREUS

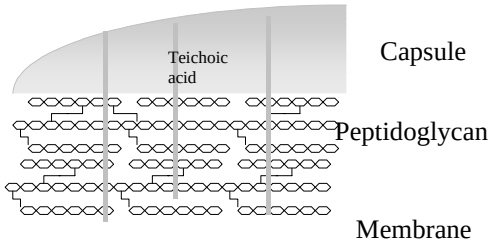
2.1.1 Laboratory diagnostics

S. aureus is a gram positive, catalase positive aerobic or anaerobic coccus showing hemolytic and large yellow colonies. The laboratory diagnostics is based on culture and biochemical tests: typical morphology, positive coagulase reaction, fermentation of mannitol and trehalose, and production of heat stabile nuclease (thermonuclease). The ability of coagulase to clot plasma is the most widely used method for identification. A four–hour tube coagulase test with reconsituted plasma is definitive, and a slide test for bound coagulase is a means of rapid screening for species identification (130). Latex agglutination tests, such as Slidex Staph Plus (Biomérieux, France) and Staphaurex Plus (Murex Biotech, England), consist of latex particles coated with IgG (bound by protein A), fibrinogen (bound by clumping factor), and IgG for *S. aureus* specific antigens. The thermonuclease can be detected using a metachromatic agar diffusion procedure and DNA toluidine blue agar (143).

2.1.2 Cell wall

The outermost layers of pathogens are important in the infection process (6). Most *S. aureus* isolates are covered by a polysaccharide capsule. Capsular polysaccharides can be classified into eleven different serotypes. Beneath the capsule *S. aureus* harbors a typical gram positive cell wall (86). The gram positive cell wall differs from that of gram negative bacteria in two major characteristics: a gram positive cell wall has a thicker and highly crosslinked peptidoglycan layer, and it lacks the outer membrane (12, 18, 220, 287) (Figure 1).

Figure 1. Schematic presentation of *S. aureus* cell wall.



The peptidoglycan consists of glycan strands of N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) disaccarides, crosslinked by tetrapeptides consisting of L-alanine, D-glutamine, L-lysine, and D-alanine (86). In S. *aureus,* a pentaglysine inter-bridge links the tetrapeptide units of adjacent glycan strands. *S. aureus* produces four penicillin-binding proteins, PBP1-4, involved in the cell wall peptidoglycan assembly (141). The biological activity of these native PBPs is similar to that of serineproteases, and they act as transpeptidases in the crosslinking of the glycan chains (181, 299). PBP2

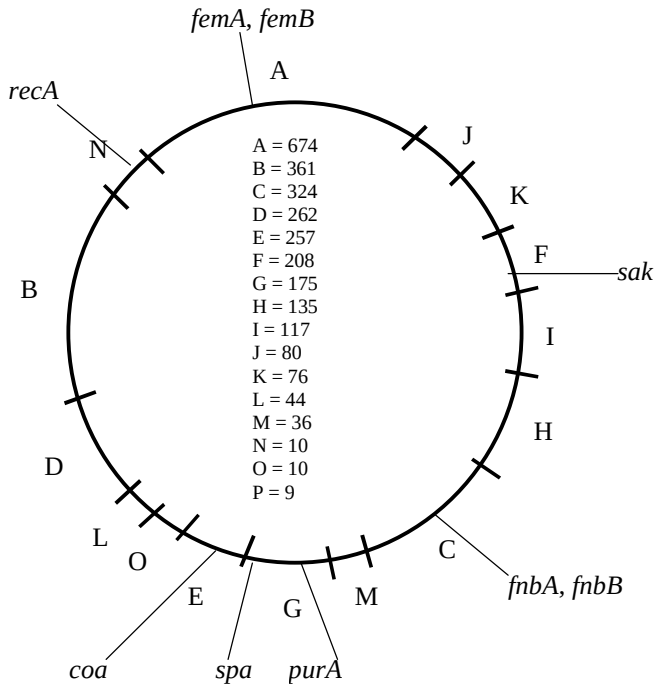
is a bifunctional protein which, in addition to transpeptidase activity, also acts as transglycosylase (91). PBPs bind effectively to beta-lactam antibiotics, and in the presens of these agents, the cell wall assembly is discontinued.

Another common feature in the gram positive cell wall is teichoic acid, a carbohydrate-phosphate polymer covalently linked to MurNAc (12). Teichoic acids bind divalent cations and possess antigenic properties (146).

2.1.3 Genome

The genomic positions of various genetic markers of *S.aureus* have been localized by creating physical maps of the genome. The development of *S. aureus* chromosomal maps began through definition of three linkage groups consisting of nine auxotrophic markers and a novobiocin resistance marker on *S. aureus* NCTC 8325 (211). By inclusion of a large number of additional markers, and the use of pulsed field gel electrophoresis (PFGE) and subsequent hybridization with available probes, the physical map of *S. aureus* NCTC 8325 continued to be more and more precise (116, 210, 295) (Figure 2). However, until data from genome sequencing became available, the mutual distances between genetic markers within each PFGE fragment were unknown.

Figure 2. *S.aureus* NCTC 8325 physical map. Adapted from (205). *SmaI* restriction fragments A-P, their sizes in kilobase pairs, and examples of identified genetic markers.



Knowledge of bacterial structures and functions has increased and will further increase owing to recent advances in genome sequencing and in other genome-wide analysis. To date, genomes of seven *S. aureus* strains have been elucidated or are soon to be completed, COL, NCTC 8325, N315, Mu50, MW2, UK EMRSA-16, and MSSA 476, (http://www.sanger.ac.uk/Projects/S_aureus, http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl, http://www.genome.ou.edu/staph.html) (11, 139). Half of all predicted proteins of strain N315 were similar to those of *Bacillus subtilis* or *Bacillus halodurans*, and these proteins mostly coded for essential functions such as DNA replication, protein synthesis, and carbohydrate metabolism. Nearly 40% of all predicted proteins were similar to as yet unknown proteins, or showed no sequence similarity in global databases. Furthermore, some of the open reading frames (ORFs) were similar to those of taxonomically distant organisms, but not all of them differed in GC content or codon usage. However, the first sextant of the chromosome showed accumulation of ORFs with unusual codon composition. It has been suggested that this region contains exogenous genes recently acquired through lateral transfer (139).

2.1.4 Diseases

Staphylococcus aureus causes a wide variety of diseases, from mild skin infections to severe life threatening systemic infections (297). It is a common cause of skin and subcutaneus infections, including folliculitis, furunculosis, cellulitis, mastitis, and impetigo. Recurrent abscesses of the skin and the subcutaneous tissue may be difficult to treat. The preferable tratment for folliculitis and local abscesses is surgical drainage, whereas cellulitis is usually treated with antimicrobials. Impetigo can range from mild, recurrent infections to a more severe bullous form and to the potentially life-threatening scalded skin syndrome (144). *S. aureus* is also commonly associated with postoperative wound infections, catheter-related infections, toxic shock syndrome (TSS), and food poisoning. TSS and food poisoning are toxin-mediated diseases. The common, self-limiting, food poisoning is caused by enterotoxins present in contaminated food, and is characterized by nausea, vomiting, headache, and sometimes diarrhea. The symptoms start four to five hours after consumption of contaminated food (112, 304). TSS, caused by TSST-1, is a potentially fatal condition, most commonly associated with the use of highly absorbent tampons, but also known in non-invasive *S. aureus* infections in children. The symptoms include high fever, rash, desquamation of skin one to two weeks after onset, hypotension, and involvement of multiple organ systems (63, 247, 268)

Serious *S. aureus* infections include osteomyelitis, pneumonia, sepsis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, scalded skin syndrome, and sterile site

abscesses (297). *S. aureus* pneumonia is rare in a community setting, but fairly common in a hospital setting, especially as a consequence of influenza in elderly patients (154). Acute osteomyelitis primarily affects long bones in children, whereas chronic (duration of infection >6 months) osteomyelitis is more common in adults after bacteremia, or as a complication of penetrating wounds (298). *S. aureus* sepsis most often originates in a local infection focus such as cellulitis, pneumonia, or a wound, or is related to an intravascular device (301). Complicated sepsis may hematogenously spread the infection to other organs, such as heart, bone, and joints. Annually, *S. aureus* causes 700-900 septic infections in Finland, and is the third most common causative agent of nosocomial sepsis (157). *S. aureus* endocarditis can present as right-sided endocarditis, often among intravenous drug users, as left-sided native valve infection, or as prosthetic valve endocarditis (120)

2.1.5 Carriage

Nasal carriage of *S. aureus* is one of the major risk factors for *S. aureus* infection (131). Although *S. aureus* can be found in different parts of the body, anterior nares is the primary ecological niche in humans (65, 227). In the healthy population 10-35% of individuals carry *S. aureus* persistently, 20-75% intermittently, and 5-50% never carry *S. aureus* in the nose. Proportions of nasal carriage patterns differ, depending on the study design and definitions for persistant-, intermittent-, and non-carrier (192). Other risk factors for *S. aureus* infection include age, dialysis, repeated rupture of the skin, and underlying conditions, such as renal or liver disease and diabetes.

2.1.6 Virulence factors

S. aureus harbors an extensive arsenal of virulence factors contributing to its ability to propagate and spread within the human host. The form and severity of the disease result from a complex interplay between the host defense and the activities of the virulence factor repetoire of the infecting strain. Considerable knowledge exists on the contribution of several virulence factors to specific diseases. Much less is known of the conserted action of these factors, their interaction with the host, and the relative importance of each factor in infection. One virulence factor may be indispensable in some infections, but insignificant in others. According to their biological function, the virulence factors can be divided into three groups: those involved in adhesion, in host defense evasion, and in tissue penetration (Table 1). One factor can serve in one or more of these activities, and different factors are produced in different growth phases.

Most clinical *S. aureus* strains produce capsular polysaccaride of serotype 5 or 8. The capsule may inhibit binding of antibodies and thereby opsonization, and phagocytosis (264, 266). In addition, capsular polysaccharide may have a role in bacterial ad-

Table 1. Examples of *Stapylococcus aureus* virulence factors.↑= upregulation, ↓ = downregulation. Adapted from (223), table 3-1, and from (193), table 1. n.d, not determined or not reported in the literature.

Virulence factor	Gene	Regulation system	Production phase	Proposed virulence function	Reference
Clumping factor	*clfA*	sar	Exponential	Attachment	(41, 175)
Fibrinogen binding protein A	*fbaA*	agr , sar	Exponential	Attachment	(40)
Fibronecting binding protein B	*fnbB*	agr , sar	Exponential	Attachment, invasion	(45, 127, 173, 252)
Collagen binding protein	*cna*	sar	Exponential	Attachment	(21)
Coagulase	*coa*	agr , sae	Exponential	Attachment	(87, 226)
Protein A	*spa*	agr , sarA , sarS ,sae	Exponential	Host defence evasion, attachment	(42, 87, 104)
Enterotoxin A	*entA*	agr	All	Host defence evasion	(63)
Enterotoxins B-E, G-J	*entB-E, entG-J*	agr	Postexponential	Host defence evasion	(63)
Toxic shoc syndrome toxin	*tst*	agr , sar	Postexponential	Host defence evasion	(63)
Exfoliative toxin	*eta, etb*	agr	Postexponential	Host defence evasion	(144)
Lipase	*geh*	agr , sar	Postexponential	Host defence evasion	(21)
Serine protease	*spr*	agr , sar	Postexponential	Host defence evasion	(224)
V8 protease	*sasP, sspA*	agr , sar	Postexponential	Host defence evasion	(8, 127)
Fatty acid metabolizing enzyme	n.d.	agr , sar	Postexponential	Host defence evasion	(33, 178)
Penton-Valentine leucosidin	*lukF-PV, lukS-PV*	n.d.	Postexponential	Host defence evasion	
Leucosidin R	*lukF-R, lukS-R*	agr	n.d.	Host defence evasion	
Capsular polysaccarides	*cap1-8* locus	agr	Postexponential	Host defence evasion	(156)
Staphylokinase	*sak*	agr	n.d.	Host defence evasion	(8)
Hemolysins , ,	hla, hlb, hld	agr , sarA	Postexponential	Tissue penetration	(226)
Hemolysin	hlg	agr	Postexponential	Tissue penetration	(8)
Phospholipase C	plc	agr	n.d.	Tissue penetration	(8)
Metalloprotease	aur	agr	n.d.	Tissue penetration	(8)
Hyaluronidase	hysA, hal	agr	Postexponential	Tissue penetration	(8)

hesion to polymer surfaces in medical devices (180).

The role of free coagulase in the virulence of *S. aureus* is uncertain. However, since it is produced by the majority of strains and has biological function as a prothrombin activator, it is considered a probable virulence factor. The cell wall bound clumping factor, another fibrinogen binding protein, shares a significant sequence similarity with coagulase, and its role in adherence is clearer than that of coagulase (61, 175, 250).

Surface proteins needed in attachment to host tissue are expressed in the logarithmic growth phase during cell wall synthesis. These microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (212) include fibronectin binding protein, fibrinogen binding protein, collagen binding protein, and clumping factor. Protein A, another surface protein also produced during cell wall synthesis, may have a role in host defense evasion, since its biological function is to bind the IgG Fc-domaine (277). The surface proteins of gram positive bacteria have a common overall structure. An aminoterminal secretory signal peptide directs the export of the protein, and it is then cleaved off. The hydrophobic carboxyterminal remains within the cell membrane, and just outside of the cell membrane the protein is then cleaved between threonine and glycine residues in a well-conserved LPXTG motive. In staphylococci, the protein is anchored to the pentaglycine cross-bridge in the cell wall peptidoglycan. A proline-rich area spans the cell wall, and the extracellular amino-terminus contains a unique sequence that recognizes the target molecule in its environment (77).

S. aureus produces a wide variety of exoproteins, most of them during the postexponential growth phase. These proteins degrade the host tissue to nutrients required for the growth of the bacteria, and/or allow the bacteria to penetrate deeper into the host tissue (63). The majority of strains produce hemolysins, nucleases, proteases, lipases, hyaluronidase, and collagenase. Alpha-hemolysin (or alpha-toxin) is dermonecrotic, neurotoxic, and lyses mammalian cells, especially red blood cells, by forming a pore in the target membrane (19). Beta-hemolysin acts as sphingomyelinase, gamma-hemolysin has leucocytolytic activity, and it has been suggested that delta-hemolysin has surfactant or channel forming properties (63). Staphylokinase is a plasminogen activator (145). Hyaluronidase digests hyaluronic acid present in the skin, bone, umbilical cord, vitreous body of the eye, and synovial fluid. Some *S. aureus* strains produce additional exoproteins, which may have host defense evasion as their major function *in vivo*. One serine protease has the ability to cleave and inactivate IgG antibodies (224). Another function of proteases may include protection against antimicrobial peptides. It has been proposed that a fatty acid modifying enzyme (FAME) detoxifies bactericidal

fatty acids (178). Penton-Valentine leucocidin (PVL) has leucocytolytic activity. Exfoliative toxin, TSST 1, and enterotoxins A-E and G-J are potent superantigens (63, 144).

Genes for *S. aureus* virulence factors may reside in plasmids, bacteriophages, transposons, or pathogenicity islands of the chromosome. Recent sequencing of two *S. aureus* strains revealed three new classes of pathogenicity islands: TSST family islands, exotoxin islands, and enterotoxin islands. A considerable number of new putative virulence genes were also identified (139).

The genes coding for virulence factors are not essential for cell-division and growth, but are useful in certain situations and environments. Constitutive expression of these genes would be an unnecessary waste of energy. Instead, many virulence factors are concomitantly upregulated, whereas others, not needed at that point in time, are down-regulated (Table 1). Two major global regulator systems have been identified: accessory gene regulator (*agr*) and staphylococcal accessory gene regulator (*sar*) (8). Other regulatory genes and operons, such as *sae*, *sigB*, *ssrA-ssrB*, and *arlS-srlR* (78, 87, 138, 310), as well as environmental factors (172), also affect the regulation of virulence factors.

The *agr* regulation turns off the surface protein expression in the late exponential phase of growth, and turns on the synthesis of secreted proteins for a relatively short time period. The swich-off mechanism of the secreted proteins' expression is unknown. Two distinct *agr* gene products, generated from promoter P2, form a classical signal transduction pathway, which is activated by an autoinduction protein also translated from the same promoter. Signal transduction leads to activation of a response regulator AgrA (195). AgrA acts together with another accessory transcription factor, SarA, to further upregulate promoter P2 and another *agr* promotor, P3 (43). The transcript from the latter, RNAIII, is the actual effector in the *agr*-regulation (195). RNAIII primarily acts at the transcription level, probably through one or more regulatory proteins. It has also been shown that RNAIII forms a specific complex with leader sequences of some of the upregulated genes (177). SarA, coded for by the *sar* locus, regulates the expression of certain exoprotein genes directly, without *agr*-activity. Many such genes, as well as the intergenic region between P2 and P3 of the *agr* operon, contain an AT-rich sequence recognized by SarA (43).

2.1.7 Pathogenesis

Staphylococcal pathogenesis results from various bacterial activities mediated by virulence factors, and from the immunological response by the host. It is commonly thought that bacterial adherence to host tissue is a prerequisite for colonization and infection. This is achieved by the MSCRAMMs (212). Subsequent survival, growth, and establishment of infection depend on the ability of the bacterium to circumvent host defense. The primary host response is mediat-

ed by polymorphonuclear leucocytes (288), which are attracted by expression of adhesion molecules on endothelial cells. The cell wall components, peptidoglycan and teichoic acids, trigger signaling pathways leading to the release of cytokines (70, 106). Leucocytes and other host cellular factors can be destructed by locally acting bacterial toxins. Anti-inflammatory response is also achieved by the staphylococcal extracellular adherence protein, Eap, which inhibits the recruitment of host leucocytes by direct interaction with the host adhesive proteins ICAM-1, fibrinogen, and vitronectin (39). If not attenuated enough, however, the robust local inflammatory response may lead to the formation of an abscess. Inside an abscess, the bacteria gradually fall into a state of nutritional stress as the density of bacteria increases. At this point the autoinduction of secreted virulence factors could enable the bacteria to break out and spread to new locations (194).

In toxin mediated diseases, superantigens bind non-specifically to the major histocompatibility complex II (MHC II) and crosslink it to the variable beta chain of T-lymphocyte. Since the normal route of internalization, processing, and antigen presentation is bypassed, this unspecific binding leads to massive expansion of T-lymphocytes and production of cytokines. Superantigens also induce endotoxin hypersensitivity and bind directly to endothelial surfaces, probably causing capillary leakage through endothelial cell death or intercellular gap formation.

In invasive diseases, such as sepsis and endocarditis, staphylococci must interact with the endothelium. By using MSCRAMMs, the bacteria can adhere to damaged areas of the endothelium, or directly to the endothelial cell via the adhesin-receptor mechanism or via bridging ligands (122). The bacteria may then be phagocytized into endothelial cells (102, 196) and/or reach the underlining tissue (154). Both endothelial phagocytosis and tissue invasion elicit an inflammatory response leading to the relase of IL -1, -6, -8, tumor necrosis factor (TNF), and subsequently interferon-gamma. Leucocytes adhere to endothelial cells and increase vascular permeability.

Although *S. aureus* is primarily an extracellular pathogen, it may sometimes survive inside non-professional phagocytes, such as fibroblasts, renal cells, and osteoblasts. Intracellular survival may explain the persistent and recurrent nature of certain staphylococcal infections (222). Intracellular staphylococci often appear as small colony variants which have mutations affecting electron transport (170), and show slowly growing, non-pigmented colonies with reduced production of virulence factors (291).

2.2 METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

2.2.1 Methicillin resistance

Methicillin-resistant strains of *S. aureus* are able to grow in the presence of beta-lactams and its derivatives,

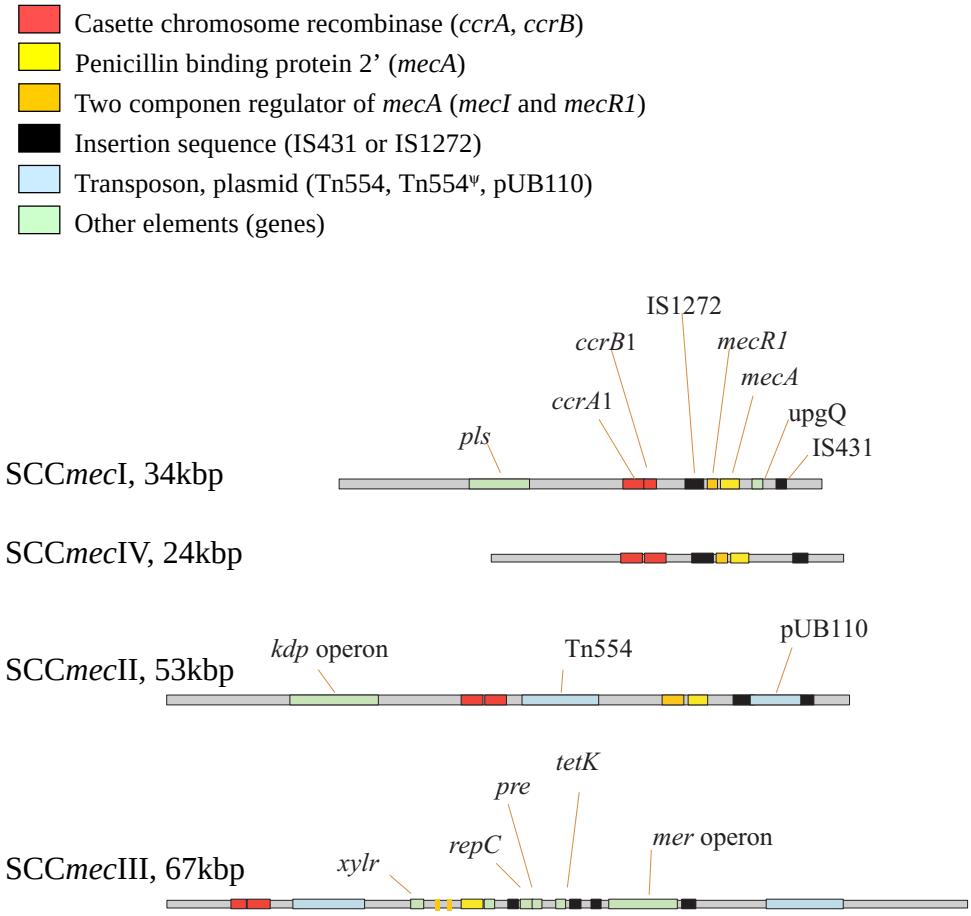
including cephalosporins and staphylococcal penicillins. Low-level methicillin resistance can result from the production of large amounts of beta-lactamases, or increased production and/or modified penicillin-binding capacity of normal PBPs (168, 269). Such borderline resistant *S. aureus* strains (BORSA) seldom have minimal inhibitory concentrations (MIC) of methicillin exceeding 16 µg/ml, and their clinical significance is thought to be limited. The challence is to differentiate BORSA strains from the true methicillin-resistant *Staphylococcus aureus* (MRSA) strains (35).

The most clinically relevant and the most prevalent form of methicillin resistance is characterized by production of an additional penicillin binding protein, PBP2a (or PBP2') (26, 228, 275). PBP2a has probably evolved by recombination of a penicillinase gene and a PBP gene similar to PBP2 and PBP3 of *Escherichia coli* (255). PBP2a has an unusually low binding affinity for all beta-lactam antibiotics, substituting the native PBPs and allowing continuous cell wall assembly (36, 228). However, the production of PBP2a alone is not sufficient for optimal expression of methicillin resistance (216). Native PBP2 provides the transglycosylase function of glycan chain elongation in spite of the presence of beta-lactams, and PBP2a is required for the transpeptidase function (215).

The PBP2a is inducible and coded by the *mecA* gene, which is part of an additional DNA region, staphylococcal casette chromosome *mec* (SCC*mec*), found in methicillin-resistant strains, but not in methicillin-susceptible strains (14, 128, 167). SCC*mec* is always located in the same region in the *S. aureus* chromosome, between *spa* and *purA* (137, 209). The sequence of the chromosomal region where SCC*mec* is integrated seems to be highly homologous among different strains. Four structurally different SCC*mec* types (SCC*mec* I-IV) and a few variants have been identified (Figure 3.) (117, 158, 198). All SCC*mec* types contain common features, including the *mecA* gene and part of its regulatory region, and *ccrA* and *ccrB* genes. The *mecR1*- and *mecI*-genes code for the regulatory proteins of the *mecA*, MecR1 is a signal transducing protein with a penicillin binding domain and a transmembrane domain, and MecI is a repressor protein of the *mecA* gene (140, 248, 263). *ccrA* and *ccrB* code for recombinases CcrA and CcrB of the resolvase-invertase family. CcrA and CcrB are required for site and orientation specific integration and excision of the SCC*mec* (118, 128).

Other genetic and environmental factors independent of SCC*mec* also have influence on methicillin resistance (15, 16, 34, 56, 57, 235). Insertional inactivation studies have identified several normal staphylococcal genes necessary for methicillin resistance. Mutations in these *fem* (factors essential for methicillin resistance) or *aux* (auxilliary) factors inhibit the peptodoglycan precursor for-

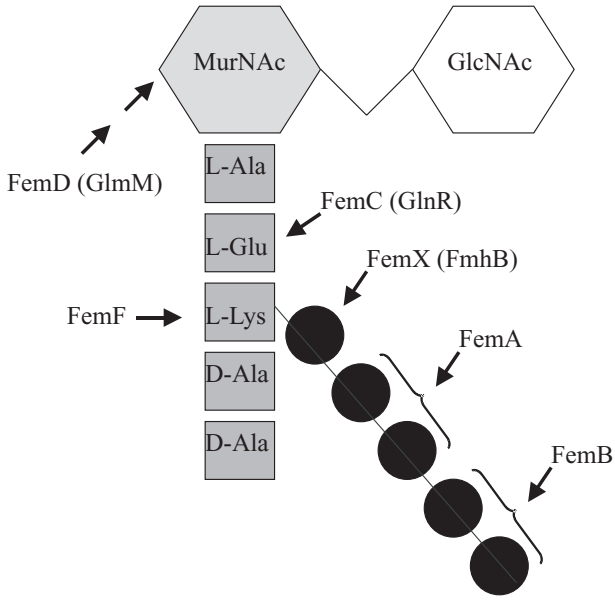
Figure 3. SCC*mec* types according to published sequences, accession numbers AB033763 (SCC*mec* I), AB063172 (SCC*mec* IV), D86934 (SCC*mec* II), and AB037671 (SCC*mec* III).



mation (Figure 4). FemA, FemB, and FemX (or FmhB) add glycine recidues in the pentaglycine interbridge between peptidoglycan strains (230, 256). Amidation of glutamate in the stem peptide is inhibited in *femC* (or *glnR*) mutants (97, 257), glucosamine-1-phosphate formation is inhibited in *femD* (or *femR* or *glmM*) mutants (124), and precursor formation is blocked at the lysine addition step in *femF* mutants (202). Other factors that may have influence on methicillin resistance include global regulators *sar* and *agr* (217), *ilm* gene coding for a lipophilic membrane protein, and genes coding for murein hydrolases.

The expression of methicillin resistance varies among strains (107, 166, 234). Strains with intact *mecI-mecR1* regulon are phenotypically methicillin sensitive, since *mecA* is effectively repressed by MecI (260). Constitutive expression of methicillin

Figure 4. Factors affecting peptidoglycan assembly and methicillin resistance.



resistance requires alterations in regulatory genes and absence of beta-lactamase regulatory genes (*blaI* and *blaR1*), which are also able to repress *mecA* (98). Clinical MRSA isolates show deletions in *mecI* and some *mecR1* regions, or mutations in *mecI* or in the promoter region of *mecA* (133, 260). Some strains express methicillin resistance heterogeneously; only a small subset of the population (10^{-3}-10^{-7}) is resistant to high methicillin concentrations. Others are homogeneously resistant, i.e. each bacterium of the population shows uniform high-level resistance (107). Strains with eagle-type resistance are sensitive in low concentrations of methicillin, but resistant in high concentrations. This type of resistance is achieved by exposing strains with intact *mecA* regulon to a high methicillin concentration. The expression of eagle-type resistance and the conversion of heterogeneous resistance to homogeneous resistance are thought to result from the same genetic factor independent of SCC*mec* and *fem* factors (134, 234).

Methicillin-resistant strains of *S. aureus* are often resistant to other antibiotic groups in addition to beta lactams. The SCC*mec* itself may carry several resistance genes generally located in integrated plasmids or transposons. Macrolide-lincosamine-streptogramin (MLS) resistance resides within Tn554, and has been linked to SCC*mec* types II, III, and IIIA (44, 265). Tetracycline resistance gene lies in pT181 in SCC*mec* type III (129). Aminoglygoside resistance genes are located either within pUB110 (*aadD*, tobramycin resistance) (28) found in SCC*mec* types IA and II or in pG01 (gentamycin resistance), which also

contains a gene for trimetoprim resistance (179). Fluoroquinolone resistance often forms during treatment because of mutation in the topoisomerase IV gene (*grlA*), the primary target of these agents in staphylococci, and high-level resistance arises through an additional mutation in the DNA gyrase gene (126, 189). Rifampin and mupirocin resistance occur because of a point mutation in target enzymes, RNA polymerase and isoleucyl-t-RNA synthethases, respectively (9, 300). High-level resistance to mupirocin arises through acquisition of a plasmid containing an additional isoleucyl-t-RNA-synthethase gene (276), but other mechanisms may also be involved (241). Strains intermediately resistant to glycopeptides (GISA) have been described in different geographic locations (110, 162, 219, 232, 253). The molecular mechanism of resistance has not been fully elucidated. However, thickening of the cell wall through accumulation of excess peptidoglycan, either by increased synthesis or by decreased turnover, seems to be common to all GISA strains (52, 103, 108). This results in the trapping of glycopeptide molecules in the cell wall, and the blocking of the access to the major target of glycopeptide antibiotics, D-ala-D-ala residue on the N-acetyl-muramicacid precursor in the cytoplasm (108). Recently, a truly glycopeptide resistant strain (GRSA) was isolated. This strain had the *vanA* gene of glycopeptide resistant enterococci. A vancomycin-resistant enterococcus was also isolated from the same infection site, suggesting that a *van* gene transfer from enterococci to staphylococci may have occurred (31).

Primary diagnosis of methicillin resistance can be performed through antimicrobial susceptibility testing recommended by National Committee for Clinical Laboratory Standards (NCCLS). The test methods are based either on diffusion of oxacillin from commercially prepared filter paper disks into the agar or on serial twofold dilution of oxacillin in an agar or broth base (187, 188). A commercially available MIC test, E-test (AB Biodisk, Sweden), is widely used in Finland. Heterogeneously resistant MRSA strains grow more slowly than do homogeneously resistant strains, and may be more difficult to detect. Addition of NaCl to the growth medium, an incubation temperature of 30 °C, and an incubation time of 48 h aid detection. Automated antimicrobial susceptibility testing systems are widely used in the USA, but not in Finland. A commercial agglutination test (MRSA Screen, Denka Seiken, Japan) based on the detection of PBP2a, and commercial agar plates with oxacillin supplements, are available for the screening for methicillin resistance (132). Definitive diagnosis is achieved using the detection of the *mecA* gene by PCR (182), hybridization, or a commercially available fluorescence test.

2.2.2 Evolution of MRSA

First MRSA strains were described in 1961 (121), in the same time period

when methicillin was introduced into clinical use. The origin of the *mecA* gene and SCC*mec* is unknown, but the *mecA* gene and the flanking regions have also been detected in other staphylococcal species. One possible source is *Staphylococcus sciuri*, since one series of studies suggested that *mecA* is a native element in *S. sciuri;* a *mecA* gene, showing 88% overall similarity with *mecA* of MRSA, was present in each of more than 100 independent isolates from different ecological sources and representing a wide variety of genotypes (49). However, the majority of these isolates showed no resistance to methicillin. The isolates which were resistant to methicillin had a further copy of *mecA*, identical to that of MRSA isolates (46, 309).

The mode of transfer of the *mecA* gene from an unknown donor to *S. aureus* has been subject to a debate. Initially, all MRSA strains were thought to be descendants of a single common ancestor, since they showed pigmentation different from MSSA strains, invariable survival capacity, and a typical resistance profile (142). Later, their clonality was studied in a more precise way by using a MRSA strain collection of 472 isolates from different geographic areas, and DNA probes to the *mecA* region and staphylococcal transposon Tn554. Twenty-nine Tn554 patterns and six different temporally ordered *mecA* patterns were found. Each Tn554 pattern associated with only one *mecA* pattern, suggesting that Tn554 polymorphism had arisen after *mecA* integration into the *S.aureus* chromosome (135). However, another study suggested that *mecA* had integrated into different *S. aureus* lineages as evidenced by size differences in the evolutionary well-conserved housekeeping proteins (183). The same protein profiles were also found in common MSSA strains. When a set of MRSA strains with these protein profiles were recently analyzed using microarrays comprising 96% of the *S.aureus* COL genome, it became evident that SCC*mec* had integrated into at least five MRSA lineages, which were highly different in their overall gene content (75). It seems, however, that the horizontal transfer of SCC*mec* is a relatively rare event, although horizontal transfer of genetic material othervise plays a major role in the evolution of *S. aureus* (75, 139)

2.2.3 MRSA surveillance

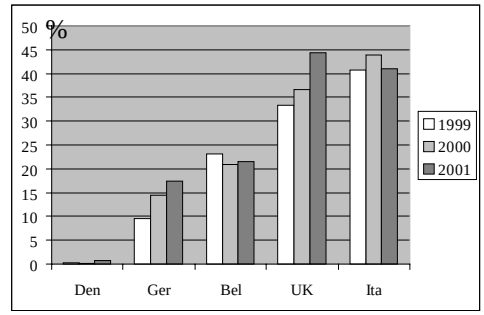
The trends of MRSA numbers and the characteristics of isolates (e.g. antimicrobial resistance) can be followed by surveillance systems (13). The surveillance may be ongoing, restricted to certain defined areas (e.g. high risk units or operations) and performed at several levels (local, national, international), and should always include feedback information to the instances which provided the data. The surveillance aims at recognizing outbreaks, monitoring the success of infection control methods, and ultimately reducing the number of MRSA and costs attributable to MRSA infections. In-

dividual hospitals often report the total number of MRSA strains isolated within a certain time period. Comparison of these reports may be difficult owing to differences or lack of denominator data. National MRSA data may be gathered as part of nosocomial infections surveillance systems, and some countries additionally require MRSA cases to be reported to national infectious diseases registers (http://www.ktl.fi/ttr/). The Nosocomial Infection National Surveillance Scheme (NINSS) in the UK involves monitoring blood stream infections and surgical site infections of more than 100 hospitals (http://www.phls.co.uk/services/nisu.htm). The National Nosocomial Infections Surveillance (NNIS) System in the USA is based on voluntary participation of 315 acute-care general hospitals (http://www.cdc.gov/ncidod/hip/surveill/nnis.htm). International initiatives include HELICS (Hospitals in Europe Link for Infection Control through Surveillance), one of whose tasks is to define and validate a methodology for pooling and analyzing nosocomial infection data from intensive care and surgery units, collected in European networks (http://helics.univ-lyon1.fr/index.htm). EARSS (European Antibiotic Resistance Surveillance System) collects antimicrobial resistance data in a standardized manner, allowing analysis of temporal and geographical resistance trends (http://www.ears.rivm.nl) (90). The SENTRY Antimicrobial Surveillance Program monitors the predominant pathogens and antimicrobial resistance of nosocomial and community-acquired infections through global sentinel hospitals. The objects covered by SENTRY include bacteremia, fungemia, outpatient respiratory infections, wound infections, and urinary infections (62).

2.2.4 MRSA in health care facilities

S. aureus is one of the major causative agents of nosocomial bacteremia (69), postoperative wound infection (190, 213), and catheter related infections (71). Most nosocomial *S. aureus* infections are endogenous, caused by the patient's own carriage strain (136). Susceptibility information on *S. aureus* bacteremias, gathered by the EARSS, indicate that the proportion of methicillin resistant strains in Europe varies from country to country, and reaches 40-50% in several areas (Figure 5). The Nordic countries and

Figure 5. Proportion (%) of *S. aureus* resistant to methicillin in European countries in 1999-2001. Den, Denmark; Ger, Germany; Bel, Belgium; UK, United Kingdom; Ita, Italy. Source: EARSS (http://www.earss.rivm.nl/index.html).



the Netherlands still have low proportions of MRSA in *S. aureus* bacteremias, from 0 to 2.5% in 1999 through 2001 (http://www.earss.rivm.nl/index.html). A recent study revealed that the proportion of MRSA among *S. aureus* blood isolates rose from 18.1 to 26.1% in San Francisco County between 1996 and 1999 (113). The US National Nosocomial Infections Surveillance System (NNIS) reported a 55.3% methicillin resistance rate among *S. aureus* strains from 5070 ICU patients in 2000, indicating an increase of 29% compared with the previous five years (1), (http://www.cdc.gov).

Prevalence studies show that MRSA is a major cause of nosocomial infections worldwide, and may account for up to 20-40% of all *S. aureus* infections (258, 293). In the USA, the prevalence of MRSA in hospitals increased from 2.4% in 1975 to 29% in 1991 (205). Similar trends have been reported in Europe. In central Europe, the prevalence of MRSA increased from 1.7% in 1990 to 8.7% in 1995 (307). In Australia, MRSA has attained endemic levels of 20-40% in the eastern cities, Sydney, Melbourne, and Brisbane, during the past decade. Other major Australian cities, Perth, Darwin, Adelaide, and Camberra, had lower rates of MRSA in the beginning of the 1990s. Recently, proportions of MRSA in these latter cities have been approaching to those of Sydney (272). Data on MRSA prevalence in Asia and Africa are scarce, but sporadic reports suggest a rapid increase of methicillin resistance among *S. aureus*: from 2% in 1988 to 33% in 1998 in two Saudi-Arabian tertiary-care hospitals (159), from 26.7% in 1990 to 70.9% in 1997 in one hospital in Taiwan (37), and from 9.8% in 1992 to 45.4% in 1998 in India, according to a surveillance study on *S. aureus* strains submitted for typing (171). Prevalence studies of short time periods revealed 27.5% and 30.5% methicillin resistance among *S. aureus* isolates in Sri Lanka and Ethiopia, respectively (85, 267)

The proportion of methicillin resistance of *S.aureus* varies among hospitals of individual countries. This variation has previously been correlated with the number of hospital ICU beds, total beds, annual admissions, or annual length of stay (100, 119, 205). Recently, this correlation has become less evident (113). Extensive use of antibiotics within a hospital may partly explain differences among hospitals in transmission rates of resistant organisms (249).

The main reservoir of MRSA in hospitals consists of infected or colonized patients. The spread of MRSA from one patient to another occurs mainly through contaminated hands of health care personnel (254). The MRSA prevention methods in non-endemic countries, such as Finland, include careful hand desinfection after each patient contact, wearing gloves and a gown when caring for MRSA patients, treatment of colonized patients, and isolation of MRSA patients and suspected MRSA positive patients

until three negative MRSA cultures have been obtained. (289, 294). In Finland, a patient is suspected to be a MRSA carrier if the patient has previously been MRSA positive, has shared a room with a MRSA positive patient, has been hospitalized outside the Nordic countries within the past six months, or has been transferred from a hospital with an ongoing MRSA epidemic (294). Countries with endemic MRSA focus their screening and isolation resources on high risk departments where the impact of MRSA spread is most pronounced (67).

The primary route of nosocomial MRSA spread between hospitals and countries is clonal dissemination of relatively few international epidemic clones. In the UK, the first epidemic MRSA (UK EMRSA-1) seemed to be similar to the epidemic strain in eastern Australia (270). This strain was later superceded by a series of other MRSA strains (UK EMRSA-2-14), and in the 1990s by UK EMRSA-15 and –16 (163, 174). UK EMRSA-15 and-16 gradually spread throughout the country, and they have also been identified elsewhere in Europe. In Germany, six different epidemic strains have gradually disseminated throughout the country (306). The Iberian and Pediatric clones, originally identified in Spain and Portugal, respectively, have subsequently been identified in several countries in Europe, Latin America, and in the USA (58, 93, 165, 229). The New York strain has spread widely in the USA and a similar strain also exists in Japan (7). Why these particular clones have been so successful in spreading remains to be elucidated.

2.2.5 Community-acquired MRSA

MRSA strains of nosocomial origin may be transmitted in the community through discharged patients or health care workers. Another possibility is that MRSA strains arise *de novo* through acquisition of SCC*mec* into the genomes of previously susceptible *S.aureus* strains.

Long-term care facilities, such as nursing homes, have been suspected as being reservoirs of MRSA. Recent studies suggest that, in the USA, long-term care residents commonly harbor antibiotic-resistant organisms, and MRSA is the most common organism found (184, 271). The rates of MRSA colonization in the nares and in wounds range from 8-53%, and 30-82%, respectively (23). European studies from the UK and Ireland suggest that the rates of MRSA colonization in long term care residents are lower, from 0.81% to 17% (50, 79, 186, 204). Even if MRSA colonization rates are high in long-term care facilities, infections of MRSA may occur infrequently. It has been suggested that facilities with endemic MRSA should perform routine surveillance of MRSA infections and uphold basic infection control practices to prevent transmission. Knowledge of the MRSA colonization status of residents is not so necessary. Only when a certain threshold of MRSA

infections is reached, should more intensive prevention measures, such as screening and isolation, be implemented until the chain of transmission is disrupted (203, 204). Prevention measures for long-term care facilities with non-endemic MRSA have not been suggested. Some studies indicate that MRSA strains isolated from long-term care residents originate in the referring hospitals, since the strains show phage types similar to hospital strains (79).

Community acquisition of MRSA has been described in rural communities with a low socio-economic status, e.g. among Australian aboriginees, American Indians, and in certain areas in New Zealand, Tonga, and Samoa (72, 95, 160, 272). Outbreaks of community MRSA have been described among intravenous drug users, members of a sports group, and families (150, 152, 239). One outbreak of gastroenteritis has also been attributed to a community MRSA (125). The overall prevalence of community MRSA among urban homeless and marginally housed adults, subjected to crowded living conditions with poor access to sanitation facilities, was 2.4%. However, the urban poor who did not have known MRSA risk factors had an MRSA prevalence similar to that of the general population (38). Household pets may also carry MRSA (151), and transmission to humans has been suspected (32). Reports on MRSA in the community are conflicting. MRSA colonization in healthy individuals without MRSA risk factors and health-care facility contacts seem to be rare (4, 237, 249, 259). On the other hand, MRSA caused the deaths of four healthy children without any known risk factors (29), and certain patient groups are commonly colonized with MRSA prior to hospital admission (72, 147, 238). These observations warrant wider investigations on the frequency of, and risk factors specific for, community acquisition of MRSA. In contrast to MRSA strains found in long-term care residents, other community-acquired MRSA usually show antibiotic resistance patterns and genotypes differing from those of hospital isolates (3, 95).

2.2.6 Molecular typing of MRSA

Molecular typing through phenotypic or genotypic methods aims at defining whether a group of strains of a single species is clonal, i.e. arises from a common precursor. For MRSA, this information is needed for various purposes. In an outbreak investigation, an increased number of spacially and temporally related strains are typed in order to identify whether the outbreak is due to the spread of a single strain. Molecular typing provides information on changes in the MRSA population during long-term surveillance, after implementation of infection control measures, or after a change in antibiotic prescribing policy. Typing is also used to deduce evolution and global spread of MRSA strains. A large number of typing methods have been developed over the past decades. The choice of the typing method depends

Table 2. Basic principles of methods used for typing of MRSA in this study. Adapted from (221)

Method	**Target**	**Principle**	**Outcome**
Phage typing	Cell surface components	Infection of strains with phages, differentiation by ability of phages to lyse distinct strains	Lytic plaques on bacterial lawn on plate
Pulsed field gel electrophoresis	Whole genome	Total genomic digestion with infrequent cutters, followed by electrophoresis with periodic changes in the orientation of electric field	Restriction fragment length polymorphism of total genomic DNA
Ribotyping	rRNA genes	Total genomic digestion, followed by Southern hybridization with rDNA probe	Restriction fragment length polymorphism based on the size and number of fragments that contain rRNA genes
HVR-hybridization	Hypervariable region within mec DNA	Total genomic digestion, followed by Southern hybridization with probes recognizing the hypervariable region	Restriction fragment length polymorphism based on the size and number of fragments that contain HVR-sequence
Multilocus sequence typing	Seven housekeeping genes	Sequencing of each gene	Nucleotide sequence

on the purpose of the analysis (outbreak investigation, surveillance, evolution studies), and on the biological performance and technical efficiency of the method (283) (Table 2). An optimal method for all purposes of typing should show high typeability, technical reproducibility, ease of interpretation of results, and low cost. For outbreak investigations the general availability of the method and ease of performance are of practical importance. The discriminatory power should be high for outbreak investigations, but less high for population studies, otherwise the clonal relationships may remain obscure (74). Reproducibility is particularly important if the typing results are (electronically) stored and later compared with new results.

Phenotypic typing methods characterize the strain relations indirectly through the expression of different genes. The results are often influenced by environmental factors. Therefore phenotypic identity or similarity is not always due to genomic identity, and genomic identity is not always reflected phenotypically.

Phage typing is one of the oldest methods used for discrimination of MRSA strains; the first set of phages was established over 50 years ago (208), and phage type data on historical *S. aureus* strains are available (82, 303). Phage typing is based on variable capabilities of different bacteriophages to lyse different MRSA strains (207). Although an international set of phages for *S. aureus* and an additional set for MRSA have been established, and standardization has been attempted, this method shows considerable inter-laboratory variation and variable typeability, and requires technical expertise (10, 164). However, it is rapid and usually sufficiently discriminative for short-term outbreak investigations.

Antimicrobial sensitivity testing has been a useful adjunct to MRSA typing in short outbreaks caused by multiresistant strains, since the antibiogram is often the first typing result available (231). Different patterns predict different sources of infection. However, the antimicrobial susceptibility pattern of an individual strain may change during treatment (126), or because of antibiotic selection pressure in hospitals.

Multilocus enzyme electrophoresis (MLEE) is based on electrophoretic mobility of several proteins essential for cell viability and growth (245). This method is not sufficiently discriminative for outbreak investigations, but instead provides information on population studies. Other phenotypic typing methods, such as biotyping, serotyping, polyacrylamide gel electrophoresis (PAGE) analysis of released or cell wall bound proteins, and immunoblotting, can be used as additional methods (244, 261). However, these methods are not often used because of lack of appropriate reproducibility or discriminatory power.

Genotypic typing is based on the analysis of a chromosome or extrachromosomal DNA, allowing direct comparison of genotypes between

strains. Analysis of plasmid profiles or restriction fragment analysis of plasmids has been used in outbreak investigations. Although the method is technically simple and feasible in most laboratories, its use is restricted because of its variable typeability and discriminatory power.

The restriction of chromosomal DNA with frequently cutting enzymes and subsequent agarose gel electrophoresis provides a discriminative fingerprint of the whole chromosome. Owing to the large number of fragments, the interpretation may be subjective and therefore non-reproducible. The interpretation can be improved by Southern blotting the DNA fragments to membrane and by highlighting a subset of the fragments with specific probes (278). The probes must include both conservative (to provide typeability) and variable (to retain discrimination) sequence areas. Preferably the target sequence should occur in multiple locations within the chromosome. DNA sequences meeting these criteria among MRSA strains include ribosomal RNA operons, Tn554, IS256, and IS 257/IS431. Individual hybridization with multiple probes, each recognizing a single-copy gene, such as those for most virulence factors, can also be used, but this approach is fairly laborious.

Several typing methods based on polymerase chain reaction (PCR) have been developed. Specific genes with polymorphic repeat regions, such as *coa*- and *spa* genes, have been used as targets for PCR amplification (80, 111). Additional discrimination can be achieved by subsequent restriction fragment length polymorphism (RFLP) analysis. PCR amplification of a tandem repeat region of the extracellular part of staphylococcal coagulase, and subsequent *Alu*I digestion, results in moderate discrimination of strains (92, 261). The variation in the protein A repeat units is also a target for typing. Changes in the protein A repeat region may, however, occur more rapidly than the entire genome evolves, and thus be an unsuitable target for purposes other than outbreak investigation (280). The ribosomal RNA sequences and the spacer sequence between 16S and 23S rDNA have also been targets for PCR-based typing. Another approach is to use as PCR targets polymorphic non-coding repetitive (ERIC or REP) sequences scattered around bacterial genomes (59). Arbitrarily primed PCR (AP-PCR) and randomly amplified polymorphic DNA analysis (RAPD) are based on short primers and low-stringency amplification conditions. Amplification of random sequences forms a PCR-pattern shared by identical strains. Low-stringency PCR conditions may reduce the reproducibility of this method, and it is seldom suitable for ongoing epidemiological surveillance, unless sophisticated equipment, high techical skills, and means for computerized data storage and interpretation are available (201). Amplified fragment length polymorphism (AFLP) is a PCR-typing method recently adapted for discrimination of

bacteria (292). In this method, chromosomal DNA is first digested with frequent cutters, specific adapters are then linked to the resulting fragments, and a subset of the fragments is selectively amplified using primers recognizing the adapter sequence and extending the original fragment by one to three selective bases. The large number of fluorecently labeled amplicons is finally separated by electrophoresis.

Binary typing is based on differential hybridization of up to 12 selected probes with different MRSA strains. For each strain analyzed, 12 "yes" or "no" (1 or 0) results are obtained (284). The suitable probes, hybridizing only with a subset of characterized MRSA strains, were initially identified by RAPD (285). The method has proved to be useful in the characterization of the genetic diversity of MRSA clone, as well as in detecting nationwide spread of one MRSA clone (282, 286).

Pulsed field gel electrophoresis (PFGE), introduced in 1984 (243), is regarded as the "gold standard" method for distinguishing MRSA strains. In this method, chromosomal DNA is digested with infrequently cutting enzymes having recognition sequences of six to eight bases in length. The digestion yields large fragments of DNA (20-800kb), which cannot be separated in conventional agarose gel electrophoresis. In PFGE, the direction of the electric field periodically changes, which allows separation of DNA fragments of up to 10 Mb in size. The separation is primarily based on the time needed for reorientation, longer for larger than for smaller fragments, instead of the speed of migration.

Comparison of whole genome sequences would be the ultimate and the most stringent typing method for any bacterium. However, for the purposes described above, whole genome sequencing is not feasible. Instead, identification of regions with both conservative and variable sequencs is needed. The degree of variation depends again on the purpose of typing. For population analysis of MRSA, multilocus sequence typing (MLST) has been used successfully (161). MLST is based on the sequencing of seven "housekeeping" genes. A distinct number is assigned to each different sequence of the same allele, and the allelic profile of the seven genes defines a sequence type (ST). Isolates with identical sequence types are considered clonal with a high degree of accuracy (74). For outbreak investigations, sequencing a single discriminatory locus, such as short sequence repeats (SSR) in *spa* or *coa* genes (80), may be more convenient and less costly.

Data storage and interpretation issues are essential as massive volumes of genotypic and phenotypic data may be generated in both short-term and long-term approaches. In addition, international comparison of MRSA strains has become increasingly relevant because of the pandemic spread of MRSA strains. Despite established interpretation criteria (262), availability of sophisticated software

packages, and several inter-laboratory standardization initiatives for PFGE (46, 281), inter-laboratory reproducibility is difficult to achieve. Although software which transform bands mathematically to numerical values do exist, PFGE and other banding pattern based methods are prone to bias due to manual selection of bands. Therefore, databases based on binary output or sequence of numbers or letters would facilitate international comparison of strains.

3. AIMS OF THE STUDY

The purpose of this study was to investigate the molecular epidemiology of methicillin-resistant *Staphylococus aureus* in a low-prevalence country. The phenotypic and genotypic characteristics of Finnish MRSA isolates were analyzed in order to gain new insights into the short-term and long-term evolution of MRSA. The specific aims were:

1. To elaborate a MRSA typing scheme suitable for continuous nationwide MRSA surveillance in Finland.

2. To analyze trends of MRSA occurrence in Finland in the 1990s and to analyze molecular characteristics of isolates in order to identify those linked to epidemics.

3. To estimate the proportion of isolates of community-acquired MRSA in Finland, and to determine if any molecular type associates with community acquisition.

4. To recognize internationally spread MRSA clones in Finland, to search for molecular traits linked to the epidemic spread of MRSA, and to analyze the clonality of the most common MRSA strains encountered in Finland.

4. MATERIAL AND METHODS

4.1 NATIONAL MRSA SURVEILLANCE

Until 1995, data on the annual numbers of MRSA isolations were collected voluntarily on a weekly basis and were available from the major microbiology laboratories, which included those of all central and university hospitals, as well as the major private laboratories. Since 1995, microbiology laboratories have reported MRSA isolations, as well as all blood and cerebrospinal fluid isolations of *S. aureus*, to the National Infectious Disease Register at the National Public Health Institute (KTL). In this register, the time interval within which *S. aureus* isolates from the same person are interpreted as one case is 36 months. The following data concerning each isolate are reported to the register: age and gender of the patient, health care institution, date of isolation, and source of positive culture.

4.2 MRSA STRAIN COLLECTION

Since 1992, all microbiology laboratories have been asked to send all MRSA isolates to the Laboratory of Hospital Bacteriology at KTL. The present study reports the analysis of this population-based MRSA collection from 1992 to 2001 (Table 3). Eighteen internationally characterized epidemic MRSA strains were used as controls. These strains were obtained from the Harmony group (http://www.phls.org.uk/International/Harmony/Harmony.htm), and included type strains for Belgium EC-1 (97S96), Belgium EC-2 (97S99), Belgium EC-3 (97S101), France A (162), France B (97121), France C (10828), Greece 1 (3680), Spain E1 (5), N. German I (134/93), Hannover III (1000/93), Berlin IV (825/96), UK EMRSA-1 (NCTC 11939), UK EMRSA-3 (M307), UK EMRSA-15 (90/10685), UK EMRSA-16 (96/32010), Pediatric clone (HDE288), Brazilian clone (HSJ216), and Iberian clone (HPV107).

4.3 EPIDEMIOLOGICAL BACKGROUND DATA

4.3.1 Hospital contacts

For publication III, the data on previous hospitalization periods of MRSA positive persons were retrieved from the National Hospital Discharge Register (HILMO) at the National Research and Development Centre for Welfare and Health (STAKES). HILMO is a civil register comprising comprehensive health-care records provided by all hospitals and health-care centers in Finland, including outpatient surgery. Each report to the register includes patient identity information, admission and discharge dates, the code of the health-care provider, the type of service, speciality, the place (home or institution) from which the patient came to the institution, and the date of the surgical procedures.

For MRSA positive persons regarding whom no HILMO reports

Table 3. MRSA isolates and methods used in the study.

Public-ation	Focus of study	Selection criteria for Finnish isolates (number of isolates studied)	Methods used
I	MRSA trends and identification of epidemic strains	All isolates, one per person, isolated in 1992-1997 (891)	• Antimicrobial susceptibility • Phage typing • PFGE and ribotyping for a subset of strains
II	Molecular traits for epidemic spread	All sporadic isolates from year 1995 (47), and representatives of intra- and interhospitally spread strains (25) from 1992-1999	• Ribotyping • Phage typing • Antimicrobial susceptibility • HVR hybridization for representatives of each ribotype
III	Community-acquired MRSA	All isolates, one per person, from 1997-1999 (526)	• PFGE • HVR hybridization for a subset of strains • Phage typing • Antimicrobial susceptibility • Search for hospitalization periods from the hospital discharge register (HILMO) and by a questionnaire
IV	Clonality and evolution of MRSA	Representatives of the most common strains in 1997-1999 (14)	• MLST • Mec regulatory region-PCR • HVR sequencing

could be found, additional background information was collected by sending questionnaires to infection-control nurses at the relevant health-care facilities. The information collected included: 1) whether the MRSA-positive person was a patient or a staff member, 2) whether the specimen was taken on a clinical or a screening basis, and 3) whether the screening sample was taken because of a hospital contact abroad or because of an epidemic situation.

4.3.2 Patient days

Annual patient days in health care were retrieved from public HILMO (Net-HILMO, http://info.stakes.fi/net-tihilmo), with no access to client specific information. A patient day was defined as 24 h during which a patient occupied a bed in a health-care institute. The admission and discharge days together counted as one patient day.

4.4 ISOLATION OF DNA, AND PRIMERS USED

Total genomic DNA was prepared for all PCR-methods, ribotyping, hypervariable region hybridization, and MLST. DNA was purified using a guanidium isothiocyanate method (218) (Publication I-III), a Qiagen Tissue Purification Kit (Qiagen, Germany), or a method using phenol chloroform elution (101) (Publication IV).

The primers used in the study (Table 4) were purchased from Amersham Pharmacia Biotech, England.

4.5 IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING OF MRSA

On reception at the Laboratory of Hospital Bacteriology, all isolates suspected of being MRSA were verified for oxacillin resistance by disk diffusion and MIC-tests (see 4.5.1) and/or by *mecA*-PCR (see 4.5.2). Initially, *S. aureus* species identification was verified only if the species identification was suspected of being incorrect: an atypical or mixed culture, or nontypable by *S. aureus* phages. The identification methods included Slidex agglutination test (BioMérieux, France), *nuc*-PCR (see 4.5.2), coagulase test (130), API Staph (BioMérieux, France), API ID 32 Staph (BioMérieux, France), and biochemical tests. Since 2000, all isolates have been tested routinely for correct species identification and methicillin resistance by *nuc*- and *mecA*-PCRs, respectively.

4.5.1 Antimicrobial susceptibility testing

The antimicrobial susceptibilities were tested by the disk diffusion method after overnight incubation at 37°C on Muller-Hinton agar plates according to the guidelines recommended by the NCCLS. The antimicrobials tested included gentamicin, tobramycin, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, rifampin, fucidic acid, trimethoprim-sulfamethoxazole, tetracycline, mupirocin, and vancomycin. Oxacillin resistance was determined by the disk diffusion method after 24-48 h growth at 30°C. MIC of oxacillin was determined by the E-test according to the manufacturer's instructions (AB Biodisk, Sweden).

4.5.2 *mecA*-PCR and *nuc*-PCR

mecA-PCR and *nuc*-PCR were performed either separately as described earlier (24, 182) or as a multiplex application. A 50 µl volume of PCR reaction mixture contained 1 µl of purified genomic DNA, 50 pmol of each of the *nuc*- and *mecA*-primers, 0.2 mM of dNTP, 1 U of DynaZyme polymerase (Finzymes, Finland), and 5 µl of 10x buffer containing 15 mM of $MgCl_2$. The PCR amplification protocol included initial denaturation at 96°C for 10 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 46°C for 30 s, and extension at 72°C for 2 min, and final extension at 72°C for 5 min. The resulting amplicons were run in a 1.5% Sea Kem agarose gel (BMA, USA) at 90V for 1 h.

Table 4. Primers used in the study

Primer	Sequence 5'→3'	Reference
mecA, forward	AAAATCGATGGTAAAGGTTGGC	(182)
mecA, reverse	AGTTCTGCAGTACCGGATTTGC	
nuc, forward	GCGATTGATGGTGATACGGTT	(24)
nuc, reverse	AGCCAAGCCTTGACGAACTAAAGC	
arcC-Up	TTGATTCACCAGCGCGTATTGTC	(73)
arcC-Dn	AGGTATCTGCTTCAATCAGCG	
aroE-up	ATCGGAAATCCTATTTCACATTC	
aroE-Dn	GGTGTTGTATTAATAACGATATC	
glpF-Up	CTAGGAACTGCAATCTTAATCC	
glpF-Dn	TGGTAAAATCGCATGTCCAATTC	
gmk-Up	ATCGTTTTATCGGGACCATC	
gmk-Dn	TCATTAACTACAACGTAATCGTA	
pta-Up	GTTAAAATCGTATTACCTGAAGG	
pta-Dn	GACCCTTTTGTTGAAAAGCTTAA	
tpi-Up	TCGTTCATTCTGAACGTCGTGAA	
tpi-Dn	TTTGCACCTTCTAACAATTGTAC	
yqiL-Up	CAGCATACAGGACACCTATTGGC	
yqiL-Dn	CGTTGAGGAATCGATACTGGAAC	
mecI forward	AATGGCGAAAAAGCACAACA	(260)
mecI reverse	GACTTGATTGTTTCCTCTGTT	
mecR1 penicillin binding domaine, forward	GTCTCCACGTTAATTCCATT	
mecR1 penicillin binding domaine, reverse	GTCGTTCATTAAGATATGACG	
mecR1 transmembrane domaine, forward	CAGGGAATGAAAATTATTGGA	
mecR1 transmembrane domaine, reverse	CGCTCAGAAATTTGTTGTGC	
Druforward	TCTGAAGCAGCTTTAAATGATG	Publication IV
ISP3TT	TTACTTTAGCCATTGCTACCTT	(200)

4.6 TYPING METHODS

4.6.1 Phage typing

Phage typing was performed with an international set of phages (207), purchased from Statens Serum Institut, Copenhagen, Denmark, at 1x and 100x routine test dilutions, both with and without heat treatment, 55°C for 3 min, of the bacteria (53). The phage pattern was defined on the basis of the weakest test dilution that produced clear lytic reactions. A minimum of 20 lytic plaques (++ reaction) was required to define a positive reaction for each phage.

All isolates included in this study were typed by phages. In publication I, the identification of epidemic strains was primarily based on phage typing.

4.6.2 Ribotyping

Ribotyping was performed as described in publication I. Briefly, genomic DNA was digested separately with one to three restriction enzymes, *Hind*III, *Eco*RI, and *Cla*I (Boehringer Mannheim, Germany). DNA fragments were separated by electrophoresis, transferred to nylon membrane (Boehringer Mannheim), and hybridized with digoxygenin labeled plasmid pKK3535 containing the *rrn* operon of *Escherichia coli* (25). Digoxygenin labeled DNA marker III (Boehringer Mannheim, Germany) or *Mlu*I digested *Citrobacter koseri* (94) was used as the molecular weight standard. A difference of one band in a ribotype, as detected by visual analysis, was considered to represent a new type. An arbitrary identification letter was assigned to each ribotype obtained by different enzymes.

In publication I, ribotyping was used to characterize further the isolates suspected of being linked to epidemics, and those nontypable by phages. In publication II, ribotyping by *Eco*RI was used for genomic characterization of all isolates with unique phage patterns (sporadic isolates) from the year 1995. In publication III, ribotyping was used to verify the relatedness of isolates from the years 1997-1999 if results from PFGE and phage typing were ambiguous.

4.6.3 Pulsed field gel electrophoresis (PFGE)

The genomic DNA was prepared in agarose blocks as described previously (89, 209) with slight modifications described in publication I. Briefly, a 0.5 ml volume of logaritmic phase cells and an equal volume of 2% SeaPlaque agarose (FMC Bioproducts, USA) were mixed and solidified. The DNA purification protocol included lysostaphin and proteinase K treament, followed by washing steps with phenylmethylsulfonyl fluoride and TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Restriction fragment length polymorphism following *Sma*I-digestion was detected with Chef DR III, Chef Navigator, or Chef Mapper (Bio-Rad, USA) for 24 h, on 1% SeaKem agarose gel (FMC BioProducts, USA) at 6 V/cm with initial and final switching times of 10 s and 60 s, respectively. A Lambda Ladder PFG marker (New England BioLabs, USA) was

used as the molecular weight standard. Since 2001, the Harmony PFGE protocol has also been used for analysis. In the Harmony protocol the chromosomal DNA fragments are separated by two phases: phase 1 for 10 h, with the initial and final switching times of 5 s to 15 s; and phase 2 for 13 h, with 15 s to 60 s, respectively. The molecular weight standard in the Harmony protocol is *S. aureus* NCTC 8325. Otherwise the method was performed as above. PFGE profiles differing by fewer than four bands were interpreted as identical or closely related (262)

In publication I, PFGE was used to characterize further the suspected epidemic strains and the isolates nontypable by phages. In publication III, all isolates from the years 1997-1999, showing differences in phage typing, were typed by PFGE.

4.6.4 Hypervariable region (HVR) hybridization

The genomic DNA was digested with *Eco*RI and *Bgl*II restiction enzymes, and hybridized with two probes prepared from plasmid pBBB30 (233). HVR probe I is a 3.6-kb fragment that recognizes the HVR of the *mec* DNA, starting within the *mecA* gene and reaching up to IS431 *mec*. HVR probe II is a 1.5-kb fragment digested with *Hind*III from the 3.6-kb fragment. This probe recognizes the direct repeat unit (*dru*) -region and an *E. coli ugpQ*-like sequence within the *mec* DNA. The digoxigenin-labeling of the probes, eletrophoresis and blotting to nylon membrane of genomic DNA fragments, and hybridization were performed as described in publication II. Digoxygenin-labeled molecular weight marker III (Boehringer Mannheim, Germany) or *Citrobacter koseri* (94) was used as the molecular weight marker.

The HVR-hybridization was performed on representatives of different ribotypes identified in sporadic isolates from the year 1995, as well as on all epidemic and local outbreak strains identified before the year 2000 (publication II). Equally, the HVR-hybridization with HVR probe I was performed on all representatives of the most common MRSA strains from the years 1997-1999 (publication III).

4.6.5 Multilocus sequence typing

Multilocus sequence typing was performed as previously described (73). Internal fragments of seven housekeeping genes [carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*)] were amplified by PCR. The PCR products were purified, and the sequences of both strands (or occasionally of one strand only) were determined by ABI Prism 310 Genetic Analyzer using the BigDye fluorecent terminator chemistry (Applied Biosystems, UK). The forward and reverse sequencing primers for each gene fragment were the same as those used for the initial PCR. The allele numbers were assigned to

each loci by the MLST database (http://www.mlst.net) and allelic profiles, defining the sequence types (STs) for each strain, were compared with those available in the database and with those found in the literature.

The MLST was used to delineate the clonality and evolution of the most common Finnish MRSA strains identified during 1997-1999 (publication IV)

4.6.6 *mec* regulatory region PCR

Using primers and a PCR protocol described earlier (260), three different fragments within the *mec* regulatory region were separately amplified. The fragments were: 1) almost the entire *mecI* gene, 2) the penicillin binding domaine of the *mecR1* gene, and 3) the membrane spanning domaine of the *mecR1* gene. The resulting amplicons were run and detected in agarose gel.

4.6.7 *mec* hypervariable region sequencing

The direct repeat region (*dru*) between the glycerophosphoryl diester phosphodiesterase (*ugpQ*)-like sequence and IS431 was amplified by using primers Druforward and ISP3TT. The Druforward primer was designed on the basis of *mec* sequence data in the EMBL database (accession no. Y14051), and the ISP3TT primer was modified from an ISP3 primer described previously (200). The purified amplicons were sequenced by ABI 310 by using BigDye fluorescent terminator chemistry (Applied Biosystems, Warrington, UK). The sequencing primers were the same as those for the initial PCR.

4.6.8 Computer-assisted analysis of typing data

Computer-assisted analysis of PFGE types and ribotypes was performed by BioNumerics, version 1.0 or 2.0 (Applied Maths, Belgium), using Dice coefficient and UPGMA (unweighted pair group method using arithmetic averages).

4.7 DEFINITIONS AND NOMENCLATURE OF STRAINS

Strain types were identified on the basis of the phage pattern (publications I and II), ribotype (publications II and III), and PFGE type (publication III). The strain types of two isolates were considered to be the same if the phage types were similar and the ribotypes were identical and/or the PFGE types differed by fewer than four bands. Multiresistance was defined as resistance to more than three antimicrobial groups other than beta-lactams, and multisensitivity as resistance up to three antimicrobial groups other than beta-lactams.

A sporadic strain of MRSA was defined as a strain isolated from one person only and displaying a unique antimicrobial susceptibility and/or strain type (publications I-IV). MRSA isolates sharing the same strain types and isolated from two or more persons in the same hospital were defined as representatives of local outbreak strains (publications I and II). MRSA isolates sharing the same strain type

and isolated from two or more persons at two or more hospitals were defined as representatives of an epidemic MRSA strain (publications I and II).

In this study, a clone was defined as a group of MRSA strains the members of which are most probably descendants of a single ancestor. To be classified as members of the same clone, isolates had to have identical or single locus variant MLST profiles, or ribotypes differing by fewer than four bands, and PFGE patterns differing by less than seven bands (publications III and IV). Clone assignment by PFGE was not performed if more than seven band differences occured. A clone may contain different epidemic or local outbreak strains.

Unless an international name for a strain was known, each epidemic strain and clone was named according to the city/region where the strain was first isolated or suspected of having been imported from. For international communication, an identification code number was given to each local outbreak strain and epidemic strain.

An MRSA isolate was defined as community acquired if the MRSA-positive specimen was obtained outside hospital settings or within 2 days of hospital admission, and if it was from a person who had not been hospitalized within 2 years before the date of the MRSA isolation (publication III).

4.8 STATISTICAL ANALYSIS AND ETHICAL ASPECTS

For categorigal variables, proportions were compared by the chi-square test with Yates' correction or by Fisher's exact test, as appropriate. The means and medians of the continuous variables were compared by Student's *t* test or Mann-Whitney *U* test, depending on the sample distribution.

On the approval of the Finnish Ministery of Social Affairs and Health and of the data protection authority, the National Research and Development Centre for Welfare and Health gave us permission to use the data from the HILMO register.

5. RESULTS

5.1 ELABORATION OF MRSA VERIFICATION AND TYPING (I, II, III, IV)

Table 5 shows the methods used for the verification and typing of MRSA isolates at the KTL Laboratory of Hospital Bacteriology during the years 1992-2002. The current scheme includes species verification and methicillin-resistance verification by multiplex *mecA-nuc*-PCR, antimicrobial sensitivity testing, and typing by phages and by PFGE. Ribotyping is used in cases of ambiguity between PFGE and phage typing results (Figure 6). MLST is available for identifying the genetic background of emerging epidemic strains.

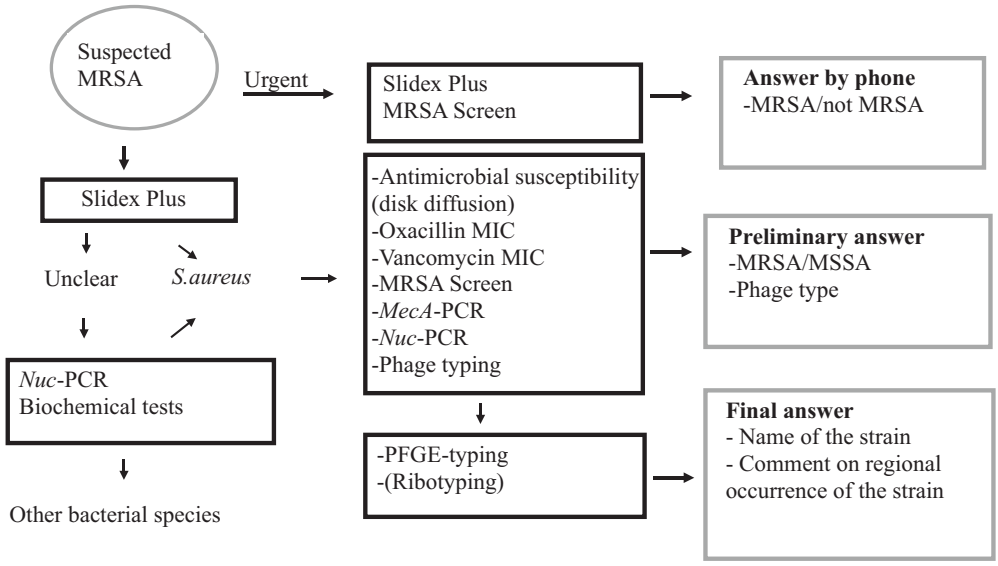
Results of MRSA verification and typing are reported to the referring laboratory and to the infection control nurse of the relevant healthcare facility. Reception of notification at the National Infectious Disease Register of each confirmed MRSA is concurrently verified.

Table 5. Elaboration of the MRSA verification and typing at the Laboratory of Hospital Bacteriology, KTL.

Purpose	Methods	Year
		92 93 94 95 96 97 98 99 00 01 02
Identification	Slidex	
	Coagulase	
	Nuc-PCR	
	API Staph, API ID 32	
	Other biochemical tests	
Antimicrobial resistance	Oxa disk diffusion	
	Oxa E-test	
	Antibiogram	
	MRSA Screen	
	MecA-PCR [1)]	
Typing	Phage typing	
	PFGE [2)]	
	Ribotyping	
	MLST	

Performed selectively on a subset of isolates, performed routinely on all isolates. 1) Until 1997, and between 1997 and 1999, mecA-PCR was performed on isolates having an oxacillin MIC of 1-6 µg/ml, and ≤64 µg/ml, respectively. 2) PFGE was performed retrospectively in 1997-1999.

Figure 6. MRSA verification and typing scheme at KTL, and the system of reporting to laboratories.



5.2 MRSA TRENDS AND EPIDEMIC STRAINS (I, II, III, IV)

On the basis of the data received from major microbiology laboratories, the number of isolates received for typing in the Laboratory of Hospital Bacteriology, and the MRSA notifications to the National Infectious Disease Register, the annual number of MRSA isolations ranged from 89 to 340 during 1981-2001 (Figure 7).

Between 1995 and 2001, 1322 MRSA isolations were reported to the National Infectious Disease Register. The median age of MRSA positive persons was 58 years (range <1-98), and 51% (674) were male. Fourty-four

Figure 7. Number of MRSA isolations in Finland during 1981-2001.

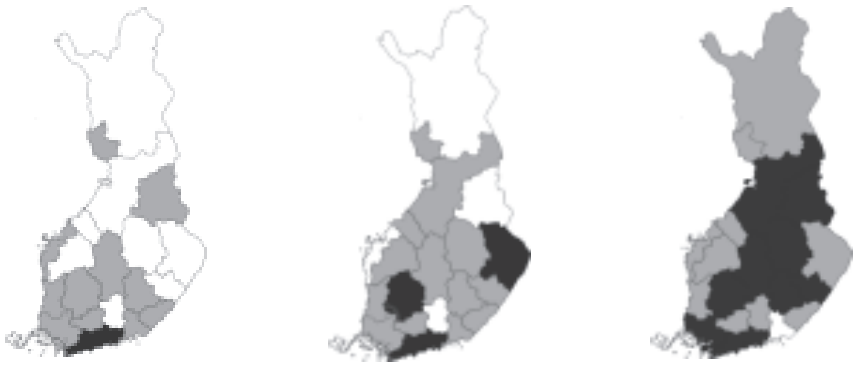
% (577) of the isolations were from persons of 60 years of age or older, and 10% (131) were from children under the age of 16. The incidence of MRSA ranged from 1.7 per population of 100,000 in 1995 to 5.0 in 2000 (Table 6). In 1995, 70% (69/89) of all notifications were received from the Helsinki metropolitan area, and 11 of 21 hospital districts reported no MRSA isolations. By 2001, all but two hospital districts reported MRSA, and the majority of notifications were outside the Helsinki metropolitan area (Figure 8). During 1995-2001, the proportion of methicillin resistance among *S. aureus* blood isolates remained at a level of 1% or less.

Table 6. Incidence and age distribution of MRSA cases in1995 through 2001

Year	Number of MRSA notifications	MRSA incidence per population of 100,000	MRSA incidence per 100,000 patient days	Median age, years	Number of persons <16 years (%)	Number of persons 65 years (%)
1995	89	1.7	NA	65	6 (7)	47 (53)
1996	108	2.1	7.11	63	10 (10)	52 (48)
1997	120	2.3	8.10	52	13 (11)	49 (41)
1998	189	3.7	12.93	51	20 (11)	54 (29)
1999	211	4.1	14.77	57	23 (11)	87 (41)
2000	261	5.0	18.47	61	25 (10)	122 (47)
2001	344	NA	NA	63	34 (10)	166 (48)

NA. Not applicable, since denominator data were not available.

Figure 8. Number of annual MRSA isolations by hospital district in 1995 through 2001. Number of MRSA isolations: □ =0, ▒ =1-10, ■ >10.



1995 1998 2001

During the period of this study, from 1992 to 2001, a total of 1847 *S.aureus* isolates were verified as MRSA and typed. Of all isolates, 79% (1457 of 1847) were classified as epidemic or local outbreak strains. The proportion of sporadic isolates during the periods 1992-1997 and 1997-1999 were 46% (345/748) on the basis of phage typing, and 11% (56/526) on the basis of phage typing and PFGE, respectively.

During 1992-2001, a total of 26 different epidemic and 12 local outbreak strains were identified (Table 7). Fourteen (54%) of the epidemic strains and 5 (41%) of the local outbreak strains were multiresistant, i.e. resistant to more than three antimicrobial groups other than beta lactams. Ribotyping by *Hind*III, *Eco*RI, and *Cla*I divided the strains into 20, 18, and 15 different types, respectively, and a combined analysis yielded 23 ribotypes. PFGE differentiated the strains into 29 distinct types and into 9 subtypes with no more than four band differences. Computer-assisted analysis of the PFGE types of the epidemic and outbreak strains revealed 8 clusters of strains with similarity values of at least 75%.

Two epidemic strains were isolated from more than 200 persons, and another seven strains caused epidemics involving 50-100 persons. The largest epidemic, caused by the strain Helsinki I, occurred in Southern Finland and involved 270 persons. The epidemics started in 1992, peaked in 1994, and declined within a few years. However, this strain is still isolated occasionally, although most of the current isolates exhibit subtype level variation, or 4 to 7 band differences in PFGE as compared with the original epidemic strain. The other highly prevalent epidemic strain, Mikkeli II, emerged in several hospitals simultaneously in 1997. The detection of its emergence was delayed, since the isolates from different parts of the country exhibited wide variation in phage types, and no epidemiological linkage between isolates from different areas was apparent. Along with comprehensive, but retrospective, PFGE analysis of isolates from 1997 to 1999, the genomic relatedness among these epidemiologically unrelated isolates was detected.

Hypervariable region hybridization with HVR probe I differentiated the epidemic and local outbreak strains into 4 types: A, B, C, and D, each of which consisted of two bands. The HVR probe II recognized one of these two bands, showing the position of *dru* and flanking sequences. Multisensitive strains had the *mec* HVR hybridization pattern A. The only exception to this was the strain Helsinki III, which was multiresistant and still had HVR-type A. HVR types B, C, and D were found in multiresistant strains. A single multisensitive strain (Turku IV) with HVR-type C was identified. Strains showing HVR type A and B were negative in *mecI*-PCR. Sequencing of the *dru* region within HVR was performed on representatives of HVR types A, B, C, and D. Sequencing confirmed that HVR types B and D had a deletion of about 1000 kb as compared

with strains with other HVR types. The *dru* sequences were highly similar within each HVR type and within HVR types B and D together.

5.3 MOLECULAR TRAITS LINKED TO EPIDEMIC SPREAD (II)

Ribotyping, with *Eco*RI restriction enzyme, of 72 strains, including all sporadic MRSA isolates from the year 1995 and the local outbreak and epidemic strains identified during 1992-1999, revealed 18 different ribotypes. The two most prevalent types, differing from each other by one band, made up 49% (35/72) of all strains. Of the sporadic strains, 45% (21/47) showed either of these types. Corresponding figures for epidemic and local outbreak strains were 64% (9/14) and 45% (5/11), respectively.

Hypervariable region hybridization with HVR probe I differentiated the 72 strains into 7 types: A, B, C, D, E, F, and G. Most of the sporadic isolates were either of HVR type A (22/47, 47%) or HVR type C (16/47, 34%). The epidemic strains were mostly of HVR type B (4/14, 29%) or type C (6/14, 43%). Of the local outbreak strains, 7 (64%) were of HVR type A.

Of the sporadic strains, 30% (14/47) were multiresistant, whereas for the epidemic, and the local outbreak strains the corresponding figures were

79% (11/14) and 45% (5/11), respectively. Multiresistance was more common among epidemic than among non-epidemic strains (11/14 *vs*. 19/58, $p<0.005$)

In a combined analysis of antibiotic susceptibility and genotype, the MRSA strains clustered into two main groups. One group included strains showing *mec* HVR hybridization pattern A combined with a variety of ribotypes and resistance to beta lactam antibiotics only. The majority of these strains were sporadic by nature. The other group included strains with *mec* HVR hybridization patterns B or C in association with the two major ribotypes. This group included both epidemic and sporadic strains.

5.4 MRSA CLONES (I, III, IV)

Of all 38 local outbreak and epidemic strains, genotyping classified 31 strains into 8 clones (Table 7). On the basis of MLST, representatives of seven of these clones were found to be identical with the internationally known *S. aureus* clones: ST 247, ST 239, ST 36, ST 22, ST 5, ST 45, and ST 12. The first six clones are also known as the Iberian, Brazilian, UK EMRSA-16, UK EMRSA-15, New York (or Pediatric), and Berlin clones. The ST12 clone was named Joensuu clone in Finland. On the basis of ribotypes and/or clustering by PFGE, six of the remaining seven strains also belong to either the Brazilian clone or to the Iberian clone. Finally, the Helsinki VIII strain was a triple allele MLST variant of Joensuu II, and may thus belong to the Joensuu clone, although the definition for a clone used in this study (see section 4.7), would

Table 7. MRSA clones and strains during 1992-2001 in Finland

Clone	Strain identification		Number of isolates	Year of first isolation in Finland	Geographical occurrence in Finland	Multiresistance	PFGE type [1]	PFGE cluster [2]	Ribotype	HVR [3]	MLST [3]
	Name	Id. no									
Brazilian	Helsinki VI	E19	15	1998	South	Yes	1	I	B.b:d	C	239
	Helsinki VII	E20	12	1998	South	Yes	2	-	C:a:e	C	239
	Lohja	E24	62	1998	South	Yes	3	III	B:b:d	C	241, 239 slv
	Kokkola	E27	78	1997	Many regions	No	4	II	A:a:e	A	8, 239 slv
	Pori II	O16	3	1993	West	No	4b	II	AN:a:e	A	
Iberian	Turku I	E6	40	1991	West	Yes	5	III	A:a:e	B	
	Turku II	E7	62	1992	West	Yes	5b	III	B:b:d	B	247
	Turku III	O8	2	1992	West	Yes	5c	III	B:b:d	B	
	Kotka	E10	32	1992	East	Yes	5d	III	B:b:d	B	
	Kerimäki	O29	34	2000	East	No	6	III	A:a:e	B	
	Tampere III	O25	15	1998	Central	Yes	7	-	A.a:e		247 slv
New York (or Pediatric)	Helsinki I	E1	270	1992	South	Yes	8	IV	I:g:e	D	5
	Koskela	O26	5	1999	South	Yes	9	IV	I:g:e	D	
	Bel EC-3	E34	33	2001	Many regions	Yes	10	IV	CH:cb:e		
	Moscow	E32	3	2000	Many regions	Yes	11	IV	A:a:e		
	Helsinki III	O3	6	1994	South	Yes	12	-	F:g:e	A	

UK EMRSA-16	Helsinki V	E5	75	1995	South	Yes	13	V	AR:af:q	C	36
	Pori I	E15	32	1993	Many regions	No	13b	V	AF:j:y	A	30, 36 slv
Mikkeli	Tampere I	E12	54	1994	Central	No	14	VI	AP:ad:c	A	
	Mikkeli I	O11	51	1993	East	No	14b	VI	AP:ad:c	A	
	Mikkeli II	E23	241	1997	Many regions	No	14c	VI	AP:ad:c	A	59 slv
	Karkkila	O37	7	2000	South	No	15	-	AP:ad:c		
Berlin	Kemi	E17	50	1996	Many regions	No	16	VII	AG:aa:r	A	45
	Kajaani	O18	11	1997	North	No	16b	VII	BB:g:r	A	
	Joensuu I	E21	8	1996	East	No	16c	VII	AW:bo:n	A	
	Pello	E28	35	2000	Many regions	No	17	VII	CC:bs:ax		
	Berlin IV	E38	13	1998	Many regions	No	18	VII	CG::aa:r		
Joensuu	Joensuu II	E22	20	1998	Many regions	No	19	VIII	AH:z:v	A	12
	Nurmes	E36	2	2001	East, South	No	20	VIII	CJ:ch:h		
	Vaalijala	O33	32	2001	East	No	21	VIII	AH:ci:e		
UK EMRSA-15	UK EMRSA-15	E30	44	1997	Many regions	No	22	-	CB:br:al	A	22
Undefined	Helsinki II	E2	36	1993	South	Yes	23	-	A:a:j	B	
(Iberia or Brazil)	Helsinki IV	O4	3	1994	South	Yes	24	I	A:a:z	C	
	Lithuania	E35	4	2000	South, East	Yes	25	I	A:a:e		
	Seinäjoki	E14	5	1992	West	Yes	26	I	B:b:d	C	
	Tampere II	E13	19	1994	Central	Yes	27	-	B:b:d	C	
	Turku IV	O9	25	1993	West	No	28	-	A:b:d	C	
Undefined	Helsinki VIII	E31	18	1997	Many regions	No	29	-	Bz:ax:h	A	New

1) PFGE types and subtypes, ≤4 bands difference
2) Cluster of strains with ≥75% similarity level by Bionumerics (dice coefficient, UPGMA, tolerance 1-2%)
3) Blank, not determined

not allow this assignment. For four clones (Iberian, Brazilian, New York, and Berlin), other strain types showing four to seven PFGE band differences as compared with epidemic and local outbreak strains have been identified.

Within one and the same clone, antimicrobial resistance patterns of different strains were mostly similar. A few exceptions were found: 1) Kokkola, Pori I, and Kerimäki strains, which showed multisensitive patterns in contrast to multiresistant patterns of all other members of the corresponding clones, 2) Pello strain, which showed two patterns, multiresistant and multisensitive, and 3) Helsinki V and Pori I strains, which constituted one clone, but showed different antimicrobial resistance patterns. Strains repeatedly isolated during long time periods (Helsinki I, Helsinki V, Kemi) showed changes in their antimicrobial resistance patterns.

5.5 MRSA IN COMMUNITY (III)

In 1997-1999, MRSA was isolated from 526 persons. Their median age was 51 years (range 0-96), and 291 (55%) were male. Of all 526 persons, 108 (21%) did not have any verified link to health-care facilities within two years before the MRSA isolation date. Their MRSA isolates were classified as community acquired. The HILMO register and the questionnaire survey showed 418 (79%) persons who had at least one contact with a hospital, and their MRSA isolates were classified as hospital acquired. The median age of the persons who did not have any contact with a health-care facility was lower than that of the persons who did (34 *vs*. 58 years, $p<0.01$).

Among the 526 MRSA isolates, the typing scheme showed 84 strain types, 56 of which were sporadic and 28 were shared by at least two persons. The distributions of sporadic (in total 56/526, 11%) and shared (in total 470/526, 89%) strain types was similar in persons with (43/418, 10%) and without (13/108, 12%) connections to health-care facilities.

Fourteen strain types, each of which was isolated from ten or more persons, represented 80% (421/526) of all MRSA isolations. Three of the 14 common strain types were more likely to be found in persons who did not have a contact with a health-care facility than in those who did have such a contact: Mikkeli clone, Helsinki VIII, and Joensuu II. Of all strains isolated from persons who had no hospital contact, 94% were multisensitive. Of the 56 sporadic strains, 41% were multisensitive.

6. DISCUSSION

6.1 ELABORATION OF THE TYPING SCHEME

Comprehensive typing of all MRSA isolates, or any particular nosocomial pathogen, has been considered unnecessary or even useless: "At best, such an expenditure of time, effort, and money provides some degree of information on the baseline genotypes of organisms in nosocomial environment. At the worst, the result is a mountain of data, which is difficult to interpret in the absence of clear driving epidemiological question" (88). However, we describe a typing scheme suitable for assisting in continuous nationwide MRSA surveillance. In addition to providing baseline information on genotypes for the whole country, and assistance in local outbreak investigations, this scheme has brought new information on the evolution and clonality of the Finnish MRSA. It is possible to conduct such intensive typing only in countries with low MRSA prevalence, and naturally this scheme needs to be subjected to constant evaluation and revision as new methods become available.

It was estimated that the proportion of sporadic strains was higher during 1992-1997 than during 1997-1999. This difference may not be real, since for the first period the sporadicity was mostly determined on the basis of phage typing. The reproducibility of phage typing is not sufficient for long-term surveillance, since, as shown by a recent study, fewer than one half of the same isolates typed on different days show identical reactions, or show reactions differing in strength only (10). The value of phage typing results for individual laboratories is also questionable. Since only strong reactions (++ or greater) are reported, a difference merely in the strength of the reactions of a group of strains may lead to incorrect interpretation. Therefore, the use of phage typing for surveillance purposes should be reconsidered. However, since phage typing performs well in short outbreak investigations (208), and is available only at KTL in Finland, it continues to be part of our routine typing scheme.

A more reliable identification of strains with similar genetic backgrounds was achieved by inclusion of PFGE in the routine typing scheme. The current widely accepted guidelines for interpreting PFGE patterns are restricted to outbreak investigations within a limited time frame (262). Our decision to use PFGE and the same guidelines also for continuous MRSA surveillance was based on several reasons: 1) PFGE was regarded as a gold standard method for the typing of MRSA; 2) the same method could be used for both outbreak investigations and surveillance; 3) computer-assisted analysis, storage of data, and comparison functions were available; 4) long-term reproducibility of other DNA-based typing methods, such as RAPD, might be more difficult to achieve (273); and 5) a less dis-

criminative method, ribotyping, was available for isolates showing PFGE patterns difficult to interpret.

MLST is not sufficiently discriminative for local outbreak investigations, where the question is whether patient-to-patient transmission has occurred. In addition, MLST is too laborious and time consuming for intensive surveillance in which every new MRSA isolate is typed. However, when a new epidemic strain emerges, MLST is a powerful tool for determining whether the same clone has been encountered previously elsewhere, and for investigating the evolutionary origin of the emerging strain. In order to optimize the global comparison of pathogenic isolates, essential information, such as geographical prevalence and distribution, virulence properties, and antimicrobial resistance data for each pathogen, should be provided. This process has already begun for MRSA, *Neisseria meningitidis*, and antibiotic-resistant pneumococci (161, 169).

The current typing scheme does not include any method for typing *mec* DNA. However, according to the present study and recent reports (107, 198, 199), comprehensive assignment of MRSA lineages requires identification of both the genetic bacground of the strain and the structural type of the associated *mec* DNA. A multiplex PCR method (197), recently developed for rapid identification of the structural types of *mec* DNA, should be evaluated, using the Finnish MRSA strain collection, for its potential inclusion in the routine typing scheme.

6.2 MRSA TRENDS AND EPIDEMIC STRAINS

Until 1997 the number of MRSA isolations remained stable, with a baseline of 100-150 annual isolations. A peak in 1994 (225 isolations) was due to the spread of the Helsinki I epidemic strain in the Helsinki metropolitan area. Some uncertainty remains about the data collected prior to 1995, since the diagnostic criteria for detecting methicillin resistance and the guidelines for weekly reporting of MRSA were not well described. Since the establishment of the National Infectious Disease Register in 1995, a continuous increase in MRSA numbers has been detected. The MRSA numbers of the first three years of the National Infectious Disease Register (1995-1997) remained within the baseline of the previous decade, but has exceeded that since 1998. This increase may be partly due to enhanced diagnostics and a general awareness of MRSA. In 1995, MRSA isolations were mostly reported from the hospital districts with the highest population densities. By 2001, MRSA isolations were reported from all over the country.

A reliable comparison of MRSA trends with other countries is difficult, since proper denominator data are rarely available for analyses. According to one year's nationwide surveillance in Switzerland, this country and Finland had a similar incidence of MRSA in 1997, 9.3 *vs*. 8.1 per 100,000 patient days, respectively (20). In Canada, the incidence of MRSA during the period 1996 to 1999 was higher than

in Finland, but both countries experienced a similar rate of increase. In Canada, the number increased from 12.7 per 100,000 patient days in 1996 to 22.6 per 100,000 patient days in 1999 (251). The corresponding figures in Finland were 7.1 in 1996 and 18.1 in 1999. The median age of MRSA positive persons was lower in Finland than in Canada, 58 *vs*. 71, respectively. However, there are differences between the Canadian and the Finnish surveillance systems. The Canadian surveillance is conducted through sentinel hospitals, most of which are tertiary-care teaching hospitals (251), whereas in Finland, all clinical microbiology laboratories report MRSA isolations. A national MRSA survey in Ireland in 1995 revealed a two-week periodical MRSA prevalence of 12.7 per 100,000 population (123). This figure is more than five-fold as compared with that of Finland for the whole year, 2.3 per 100,000 population in 1997.

Despite a reasonably good MRSA situation on the national level in Finland, numerous strains causing local intrahospital outbreaks or interhospital epidemics were identified. New strains emerged throughout the study period, and twelve strains have continually been isolated since their first appearance, although the numbers of specific strains tend to decline by time. Genetic alterations, as detected by PFGE subtype variation, were occasionally observed in strains that reappeared after the first outbreak had been successfully controlled.

Approximately one third of all MRSA strains in Finland are multisensitive. This is in contrast to elsewhere in Europe, where only 13 % of MRSA isolates, collected from 25 university hospitals in 15 countries through the SENTRY study, were multisensitive (76).

6.3 MEC HYPERVARIABLE REGION

The *mec* determinants of the 38 epidemic and outbreak strains were divided into four different types according to their hypervariable region hybridization profiles. An analysis of sporadic strains from the year 1995 revealed only three additional HVR-types, each encountered in only one strain. Previous studies have shown that hypervariable region analysis by PCR and sequencing can be used to distinguish different *mec* DNA types (191, 233, 308). Five to eighteen different HVR-types have been identified in collections of 24-254 strains on the basis of differences in either the number or the sequence of *dru* repeats (185, 191, 242, 246, 308). Consistently with the present study, the same HVR-type was often found in different genotypes. It is also possible that strains with identical genotypes harbor differences in HVR types. Five different subclones were identified among 50 isolates representing one epidemic strain in Germany (308). In the present study, the HVR types were mostly analyzed for one representative of each strain type, and therefore no conclusion can be drawn regarding the

variety of HVR types within different strain types.

6.4 MRSA CLONES AND TRANSMISSIBILITY

Two major groups of MRSA were identified on the basis of antimicrobial susceptibility testing, ribotyping, and hybridization analysis of the *mec* hypervariable region of 72 epidemic, local outbreak, and sporadic MRSA strains. One group contained mainly sporadic strains showing *mec* HVR type A, multisensitivity, and a variety of ribotypes. The other group contained strains with *mec* HVR type B or C in association with two common ribotypes, and resistance to other antibiotic groups in addition to beta lactams. The latter group contained both epidemic and sporadic strains. Although two major groups with distinct genotypic and phenotypic traits were identified, none of the characteristics were associated directly with epidemicity or sporadicity. The failure to find such an association may be due to incorrect assignment of sporadic strains. Any strain isolated from one person only and showing a unique phage type and/or antimicrobial susceptibility pattern was defined as a sporadic strain. A classification of strain types on the basis of phage typing alone was later shown to be inadequate. In addition, an MLST analysis of the most common Finnish isolates from 1997-1999 showed that, although showing variable PFGE patterns, many of the previously identified epidemic and local outbreak strains actually descended from a common ancestor. Some of the MRSA isolates originally classified as sporadic may also have belonged to these clones.

By a combination of several typing methods (phage typing, ribotyping, PFGE, and MLST), this study identified eight clones of MRSA in Finland. Six of the clones contained strains showing major MLST sequence types recently identified among MRSA strains from several different countries (74). Of a total of twelve international MRSA sequence types identified in a collection of 359 international MRSA isolates, nine (ST 5, 36, 347, 239, 241, 8, 22, 45, and 30) occurred in ten of the fourteen Finnish MRSA strains analyzed: Helsinki I, Helsinki V, Turku II, Helsinki VI, Helsinki VII, Lohja, Kokkola, UK EMRSA-15, Kemi, and Pori I. In addition, one strain, Tampere III, was a single locus variant of ST 247.

Two of the eight MRSA clones (Joensuu and Mikkeli) encountered in Finland contained strains showing different molecular epidemiology. Although these two clones were abundant among MRSA strains in Finland, the MLST sequence type ST 12 of Joensuu II is commonly found among international MSSA, but not among MRSA strains (http://www.mlst.net), (74), and ST 59, the slv of the Mikkeli clone has been thus far reported in one MRSA carrier and in few MSSA strains (http://www.mlst.net).

Patient-to-patient transmission of MRSA depends on many factors. Patients colonized or infected with

MRSA are the major reservoir in hospitals. Patient risk factors for MRSA colonization and infection include previous hospitalization, pressure sores, indwelling devices, underlying disease, and recent antibiotic use (47, 64, 236). Other factors promoting the spread of MRSA include care in high-risk departments such as burns unit or intensive care unit (47, 225), staff shortage (96), frequent patient transfer between wards and hospitals (60, 68), and antibiotic selection (249). MRSA dissemination may be reduced by appropriate infection control measures and antibiotic policies (96). However, the identification of a relatively small number of global MRSA clones among the large diversity of *S. aureus* clones suggests that strain characteristics also influence the dissemination of MRSA (http://mlst.net), (51, 54, 74). The majority of the globally successful clones were also identified in Finland. Success in transmissibility seems not to be exclusively due to SCC*mec*, since 4 of the 12 recognized MRSA STs (ST 5, 8, 22, and 45) in Finland were common among European MSSA strains from the 1990s (74), (http://www.mlst.net).

The reasons for superior transmissibility of some clones still remain unclear, although several mechanisms have been suggested. Two epidemic strains have been shown to possess better environmental survival than have three sporadic strains. The recovery capacity of all five strains declined gradually, but the epidemic strains survived three months longer, in up to approximately 1000-fold higher quantities (cfu/ml), than did the sporadic strains (296). For some strains, enhanced transmissibility may result from better colonization at the expense of reduced virulence (153), as suggested for the Canadian epidemic strain CMRSA-3 (206). CMRSA-3 exhibited significantly higher fibronecting-binding and coagulase titers than did another epidemic strain, CMRSA-1, and sporadic strains. In the case of CMRSA-3, the balance towards enhanced colonization factor production may be explained by reduced and delayed RNAIII production by the global *agr* regulator (206). CMRSA-1 did not produce alpha toxin and proteases, and had a limited profile of secreted proteins but a normal level of RNAIII production. The predominant subtype of CMRSA-1 expressed a high molecular weight glycoprotein known to prevent bacterial adhesion to fibronecting, fibrinogen, and IgG (240). However, this protein may confer novel adhesion functions, such as those related to biofilm formation, and adherence to cellular lipids (114, 115). It has also been suggested that a high number of repeats (≥7) in the polymorphic X-region of the protein A gene would predict an epidemic nature of MRSA strains. A longer X-region in epidemic strains may result in a better exposition of the Fc-binding region of protein A (81). Another study did not support this hypothesis, since no correlation was found between the length of the protein A gene repeats and the persistence of *S. aureus* colonization

(279). In the light of the present and previous studies and the fact that *S. aureus* colonization and virulence result from a complicated interplay of numerous factors, it seems that no single mechanism is responsible for the successful transmission of certain clones. Instead, these clones may harbor a wide range of mechanisms affecting transmissibility, and the mechanisms may vary between different clones and even between subtypes of individual strains (206).

Reliable distinguishing of sporadic strains from strains with evident capacity to spread is crucial in the search for molecular markers or mechanisms for enhanced transmissibility. This requires representative population-based strain collections and careful characterization of isolates. Such material is available in Finland because of the national reporting and typing systems.

6.5 MRSA IN COMMUNITY

Our study showed that, from 1997 to 1999, one fifth of all Finnish MRSA isolates came from persons who had had no contact with health-care facilities, suggesting that these MRSA isolates may be community-acquired. This unexpectedly high proportion of community-acquired isolates was identified despite a stringent definition for community acquisition (22), i.e. a 2-year time period without health-care facility contact before the MRSA isolation. However, a possibility remains that some of the MRSA-positive persons may have had health-care facility contacts before the 2-year cut-off period, and their MRSA isolates had been acquired in hospital and had persisted ever since.

Risk factors other than previous hospital stays were not analyzed in this study. Further information should be collected to develop a hypothesis on risk factors specific for community acquisition. Intravenous drug use, previous antibiotic use, and underlying diseases have previously been associated with community acquisition of MRSA (147, 176). A recent study covering 30 months identified a similar proportion (22%, 20/92) of community-acquired MRSA in a university hospital. Thirteen (65%) persons with community-acquired MRSA lacked the known risk factors (27). A known risk factor was defined as a previous hospitalization within 12 months, an underlying chronic disease, the presence of an indwelling catheter, a history of surgical procedures, previous antimicrobial therapy, intravenous drug use, or a household contact with an identified MRSA carrier.

Community-acquired MRSA can be classified into four categories: 1) discharged hospital patients and hospital staff members with MRSA; 2) nursing-home residents with MRSA; 3) MRSA transmitted to non-hospitalized patients; and 4) MRSA arising *de novo* in community (48). Our study focused on the last two categories by first identifying nonhospitalized MRSA-positive persons, and thereafter comparing their isolates with those from hospitalized patients

Of all MRSA strain types isolated from ten or more persons during 1997-1999, three were associated with community acquisition: Mikkeli, Joensuu, and Helsinki VIII. Coincidentally, all these strain types showed related MLST sequence types previously encountered mostly among successful MSSA clones, but not among MRSA clones (74). In addition, three other strain types were also frequently found among persons without health-care facility contacts: Kemi, Kokkola, and Pori. All these strain types were characterized by multisensitivity and HVR type A. Preliminary PFGE studies indicate that the strain types similar to Mikkeli, Joensuu, Kemi, and Pori were also prevalent among contemporary MSSA isolates in Finland (unpublished data). In contrast, the multiresistant strain types, representing the globally spread MRSA clones (Brazilian, Iberian, New York, and the UK EMRSA-16), were almost exclusively found in persons who had health-care facility contacts.

Recent sequencing of a virulent community-MRSA strain, MW2, which caused the deaths of four children without risk factors (29), suggested that different factors may be needed for competitive survival in different environments (11). Multiresistance may be biologically costly to maintain, and provides no additional fitness in an environment where the antibiotic selection pressure is low (5). The highly prevalent and virulent community-MRSA, MW2, grows faster, and has fewer transposons and insertion sequences than have strains adapted to the hospital environment. In addition, MW2 harbors virulence factors not found in other MRSA genomes whose complete sequences are available (139). The superantigen enterotoxin H, the penton-valentine leucocidin, a bacteriocin, and a collagen-adhesin were suggested as being partly responsible for the observed virulence (11). The virulence factors of the identified community-MRSA strains in Finland have not yet been assessed in any way. However, most of the community-acquired MRSA were multisensitive, and children were more likely to have a community-acquired than a hospital-acquired MRSA. These findings agree with those of previous reports, suggesting that nonmultiresistant MRSA is emerging as an important pathogen in the community (29, 72, 105).

6.6 HORIZONTAL TRANSFER OF *MEC* DNA

According to the present study, horizontal transfer of *mec* DNA has partly influenced the epidemiology of MRSA in Finland. This is supported by several findings.

First, we found that similar MRSA genotypes had different *mec* DNA, as assessed by *mec* hypervariable region hybridization and PCR analysis of the *mec*-regulatory region. These results are in line with previous findings (74, 75, 198) and suggest that representatives of similar genotypes may have acquired the *mec* DNA from different donors.

Second, HVR type A was found in many different genotypes, most often in those with a multisensitive antimicrobial resistance pattern, but also in some isolates of Iberian, New York, and UK EMRSA-16 clones. Other HVR types were restricted to a few clones: HVR B in one, C in two, and D in one clone. These results suggest that HVR A *mec* may transfer more easily than others. It has been suggested that one *mec* type (*mec*SCC IV) can spread to most, if not all, *S. aureus* genotypes (5). The small size of SCC*mec* IV may explain its enhanced transfer capacity. Although it remains to be clarified whether different HVR types are associated with specific SCC*mec* types, our data suggest that HVR type A may be similar to SCC*mec* IV (158, 198)

Third, two common clones of MRSA in Finland, the Mikkeli and Joensuu clones, expressed MLST sequence types usually found in methicillin-sensitive strains (http://www.mlst.net). These MRSA clones, and the Helsinki VIII strain, which is a triple allele variant of the Joensuu clone, are multisensitive, express HVR type A, and are associated with community acquisition. Furthermore, methicillin-sensitive *S. aureus* strains with PFGE types related to the Mikkeli and Joensuu clones are common in Finland. International community-acquired MRSA strains have been shown to harbor a type of *mec* DNA, which lacks resistance determinants other than the *mecA* gene, and regions known to contain mutated or partially deleted elements. This type of *mec* DNA has also been encountered in coagulase negative staphylococci (109), and it may be more capable than other *mec* DNAs of confering beta lactam resistance to community strains of *S. aureus* (11, 158). Our results support this hypothesis.

Emergence of new MRSA clones has also been encountered in Germany (305). Furthermore, strains resistant to fewer antimicrobial groups than before have also been encountered in France, and these strains have progressively replaced previous genetically different, multiresistant MRSA clones (83, 148, 149).

The *mecA* gene is widely distributed among different species of coagulase negative staphylococci (214, 274), and such strains have been detected in both nosocomial infections and healthy carriers (55, 66). Thus, donors of *mec* DNA may be available both in nosocomial and community settings.

Overall, it seems evident that the increase in the number of MRSA is not exclusively due to clonal dissemination, but that emergence of *de novo* MRSA through horizontal transfer of *mec* DNA occurs occasionally.

7. CONCLUSIONS AND CONSIDERATIONS FOR THE FUTURE

A suitable typing scheme for continuous nationwide MRSA surveillance was established. In a low prevalence country, comprehensive molecular typing, verification of methicillin resistance and *S. aureus* species, as well as antimicrobial susceptibility testing of all new isolates is feasible. Continuous feedback to laboratories and infection control nurses is provided.

MRSA trend in Finland is increasing. However, the incidence of MRSA is still low as compared with that in many other European countries and the USA. The proportion of MRSA isolated from persons without hospital contacts was surprisingly high, one fifth of all isolates.

Two epidemiologically distinct MRSA populations were found. The first population consisted of global MRSA strains, which mainly spread within and between hospitals through clonal dissemination. The second population consisted of strains genotypically related to MSSA. Some of these strains may emerge through horizontal transfer of *mec* DNA. Subsequent clonal dissemination may occur, often locally in susceptible environments, such as long-term care facilities. Some of these strains were associated with community acquisition.

The two MRSA populations may require different infection control measures. The control of global MRSA in Finland has been quite successful, as exemplified by the decline of such isolates in the largest hospital district. In contrast, increasing problems are encountered with the MRSA population consisting of strains genotypically related to MSSA. Although easier to treat, they may be more difficult to diagnose because of their low oxacillin MICs and heteroresistance. Factors influencing the emergence of such strains are unknown, but antibiotic selection pressure may play a role. If the emergence of such strains cannot be hindered, efforts should be aimed at the prevention of secondary transmission.

This study brought up several items which call for more precise research in the future. Further clinical information should be collected on persons with community-acquired MRSA in order to develop a hypothesis on risk factors specific for community acquisition. Association of HVR-types with different SCC*mec* types should be addressed. Many open questions related to the horizontal transfer of *mec* DNA remain to be elucidated. Is HVR type A associated with community acquisition? Does *mec* DNA characterized by HVR type A transfer more easily than do other *mec* DNA types? Are the *mec*-integration areas different between isolates showing MLST STs common to both MRSA and MSSA as compared with those of MSSA exclusively? Furthermore, the SCC*mec*-types in coagulase negative staphylococci should be studied in more detail.

The near future will provide new techniques for typing and for predict-

ing the epidemic capacity or pathogenicity of MRSA strains. Both structural and transcriptional differences in genomes can be revealed using microarray hybridizations. PCR or sequencing approaches may be developed for the allotyping of already identified genomic islands. The availability of complete genome sequences of several strains will result in identification of new areas important for the understanding of the adaptive fitness of MRSA strains found in different environments.

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10. ORIGINAL PUBLICATIONS